Effects of Pharmaceuticals and Personal Care Products on Different Developmental Stages in Fish

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Summary

In the last decade, environmental concerns arose due to the analytic-chemical demonstration of pharmaceuticals and personal care products (PPCPs) in the aquatic environment. Residues of numerous PPCPs occur in the ng/L to low µg/L range in surface waters. Consequently, aquatic organisms are continually exposed to them at contaminated sites. However, these low concentrations remain a cause for concern, since some of these compounds are biologically active at low concentrations, and only little is known about their effects on the environment. Pharmaceuticals as a major category of PPCPs are mostly designed to interact with cellular receptors at low concentrations to elicit specific biological effects. Their effects on non-mammalian species might be similar to those occurring in mammals, since many target receptors are conserved between vertebrates. Therefore, we hypothesize that PPCPs can have similar biological and toxicological effects in non-target species as in humans.

The overall goal of this thesis is to investigate the effects of two pharmaceuticals, the benzodiazepine diazepam and the antineoplastic agent PKC412 (protein kinase inhibitor) as well as the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) on gene expression changes and physiology of fish at concentrations near to those found or expected to occur in the environment. For the pharmaceuticals we aimed to link changes on the transcription level with effects on the organism level in order to understand their relationship and their ecotoxicological significance. The following studies have been performed:

***In vitro* study: CYP3A assay**

In a first study, an assay for measuring the activity of cytochrome P450 3A (CYP3A) was optimized for its use in different fish cell lines. Several CYP3A substrates in fish cells were found to be similarly catalyzed to those in human cells. Additionally, the established assay allows a high-throughput screening for different CYP3A inducers and inhibitors.

In subsequent studies we investigated the gene expression pattern upon exposure of zebrafish eleuthero-embryos to diazepam, and the protein kinase inhibitor PKC412, and of adult male fish (diazepam). By applying microarrays the transcription profile of the UV-filter EHMC was also studied in adult zebrafish.

The benzodiazepine derivate diazepam

The treatment of male adult fish with 235 ng/L and 291 µg/L diazepam resulted in expression changes of 51 and 103 genes, respectively. In addition, Gene Ontology (GO) analysis was performed to identify functional groups of genes of interest. At 235 ng/L diazepam, functionally identified genes fell into 57 different categories and at 291 µg/L diazepam into 40

categories. Among those categories, alteration of genes involved in rhythmic processes, especially in the circadian rhythm, is of particular significance. Furthermore, MetaCore pathway analysis showed that the circadian rhythm is the most significantly altered pathway. In order to confirm microarray results, qRT-PCR was performed for selected genes (*arntl2*, *cry2b*, *cry5*, *nr1d1*, *per1, arr3*, *gabrr1* and *hsd17β3*). The differential expression of these genes upon exposure of adult male zebrafish was confirmed in zebrafish eleuthero-embryos exposed to the same nominal concentrations of 273 ng/L and 273 µg/L diazepam.

Subsequently we analysed whether these alterations on mRNA levels are linked to physiological effects such as the swimming behaviour. At 273 ug/L diazpam the locomotor activity significantly increased in eleuthero-embryos. Additional histological analyses of testes did not indicate any significant effects, although alterations in interstitial tissue were noted. This study leads to the conclusion that diazepam-induced alterations of genes involved in circadian rhythm are paralleled by effects in neurobehaviour at high, but not at low diazepam concentrations that may occur in polluted environments.

Protein kinase inhibitor PKC412

Exposure of zebrafish eleuthero-embryos to PKC412 lead to transcriptional changes of 259 genes at 1.3 µg/L, and 511 genes at 21 µg/L. Some of the pathways affected by PKC412 were angiogenesis, apoptosis, DNA damage response and response to oxidative stress. Behavioural, developmental and physiological effects were investigated in order to analyze for correlations between altered gene-expression profiles with effects on development and physiology. The expression of circadian rhythm genes was further investigated, however, no effects on the swimming behaviour were observed. In addition, potential effects of PKC412 exposure to angiogenesis were analyzed in double transgenic zebrafish embryos Tg(fli1a:EGFP)y1;Tg(gata1:dsRed)sd2. No interference was noted based on the lack of major effects. Furthermore, effects of PKC412 on apoptosis were investigated. Apoptotic cells were found in the olfactory placodes of eleuthero-embryos at 31 µg/L PKC412. DNA damage was induced at 1.6 µg/L and 31 µg/L, but there were no significant effects on the formation of reactive oxygen species. This study leads to the conclusion that PKC412-induced alterations of gene transcripts are partly paralleled by physiological effects at high, but not at the low PKC412 concentrations expected to be of environmental relevance.

UV-filter EHMC

Adult male zebrafish were exposed to 2.2 µg/L and 890 µg/L EHMC for 14 d. This treatment led to the alteration of 1096 and 1137 genes, respectively. 708 of the significantly altered genes were regulated at both concentrations always in the same direction (up or down).

Gene Ontology (GO) analysis was performed to identify functional groups of genes of interest. At both concentrations differentially altered genes fell into 1637 different categories. Among them cellular processes, development processes and response to different kinds of stimuli (endogenous, hormone, steroid hormone) are affected at both EHMC concentrations. Additionally, we performed a pathway analysis with MetaCore. The different treatments had 33 (2.2 µg/L EHMC: 33 maps; 890 µg/L EHMC: 36 maps) maps in common. The most relevant maps include pathways involved in tissue remodelling, wound repair, DNA damage response, apoptosis and many more. To validate gene expression changes obtained in microarrays, the expression of 5 different genes (*vtg1*, *ptgds, igfbp1, rbp2a* and *gadd45b)* was analyzed by qRT-PCR in whole body, and additionally, in liver, brain and testis. In addition, the effects of EHMC on different genes involved in hormonal pathways (*vtg3*, *esr1*, *esr2b*, *ar*, *hsd17β3*, *cyp19b* and *cyp19a*) were investigated. Different trends in gene modulations were observed among whole body and the investigated tissues. Therefore, a gene expression in individual tissues varies, and must not be reflected in the whole body response.

In conclusion, it is not possible to specify a specific mode of action of EHMC, as it displayed multiple hormonal and additional effects in different tissues and the whole body. To obtain a more complete toxicological profile of EHMC, further studies are needed to relate the transcription profile to physiological effects, and to potential effects on fertility and reproduction.

The data obtained in the *in vivo* experiments demonstrated that microarray analysis could reveal new insights into the modes of action of these compounds. Gene expression changes are very sensitive and may occur at low concentrations (diazepam, EHMC), which were near to environmental relevant ones. However, links to changes in locomotor activity (diazepam, PKC412) and other cellular processes (angiogenesis, apoptosis, DNA damage and formation of reactive oxygen species; PKC412) mainly occur at concentrations up to 10,000 times higher than anticipated in the environment.

In conclusion, the data of this thesis reveals a novel and more detailed pictures of the modes of action of diazepam, PKC412 and EHMC. Toxicogenomics is confirmed to be an appropriate approach to obtain broad information on the transcriptome. Nevertheless, it was only partly feasible to link the obtained gene expression changes to physiological effects in fish. Therefore, further effort should be made on research into the link of gene expression patterns and physiological effects. Consequently, the question to what extent toxicogenomics serves as a valuable tool for the environmental risk assessment should further be evaluated.

Zusammenfassung

In den letzten Jahrzehnten wuchsen beständig Bedenken über eine mögliche Umweltgefährdung durch Rückstände von Arzneimitteln und Körperpflegeprodukten. Rückstände zahlreicher Arzneimittel und Körperpflegeprodukte in Oberflächengewässern werden regelmässig im Bereich von ng/L bis hin zu µg/L gefunden. Dadurch sind vor allem aquatische Organismen diesen Substanzen kontinuierlich an den belasteten Standorten ausgesetzt. Obwohl meist tiefe Substanzkonzentrationen vorherrschen, sind diese bedenklich, da einige der fraglichen Substanzen schon bei tiefen Konzentrationen biologisch aktiv sind. Zudem sind bei vielen Umweltchemikalien die Auswirkungen auf Nichtzielorganismen bzw. die Umwelt im Allgemeinen unbekannt. Arzneimittel wurden grösstenteils entwickelt, um schon bei tiefen Konzentrationen mit zellulären Rezeptoren zu interagieren bzw. um zu einer spezifischen Wirkung zu führen. Ihre Auswirkungen auf höher entwickelte Nichtzielorganismen (wie z.B. Fische) können folglich ähnlich zu denen in Säugetieren sein, da die meisten Zielrezeptoren auch bei diesen Organismen vorhanden sind. Aufgrund dessen wird angenommen, dass Arzneimittel und Körperpflegeprodukte die gleichen biologischen sowie toxikologischen oder zumindest vergleichbare Auswirkungen auf Fische wie auf den Menschen haben.

Das Ziel dieser Arbeit ist es, die Effekte von zwei Arzneimitteln (Diazepam und der antineoplastische Wirkstoff PKC412) und dem UV-Filter 2-Ethylhexyl-4-trimetoxycinnamat (EHMC) auf Genexpressionsebene und auf die Physiologie von Zebrafischen nahe umweltrelevanter Konzentrationen zu untersuchen. Bei der Exposition gegenüber den Arzneimitteln wurde versucht, die erhaltenen Änderungen auf der Transkriptionsebene mit Effekten auf der Organismusebene zu verbinden, um ihre umwelttoxikologische Relevanz besser zu verstehen. Folgende Studien wurden ausgeführt:

***In vitro* Studie: CYP3A Assay**

In einer ersten Studie, wurde ein Assay für die Messung der Cytochrom P450 3A (CYP3A) Aktivität verschiedener Fischzelllinien optimiert. Es wurde gezeigt, dass CYP3A Substrate sowohl in menschlichen Leberzellen als auch in Fischzellen (Zebrafisch Leberzellen, *Poeciliopsis lucida* Leberzellen, Regenbogenforellen Gonadenzellen und Fathead Minnow Epithelzellen des Anus) katalysiert werden. Zusätzlich erlaubt dieser Assay, dass mit einer hohen Durchsatzrate nach potentiellen CYP3A-Hemmern oder -Induktoren gesucht werden kann.

In den nachfolgenden Studien wurden die Genexpressionsänderungen in Zebrafischlarven nach der Exposition mit Diazepam und PKC412 untersucht. Zusätzlich zu den Larven wurden ebenso die Effekte von Diazepam auf ausgewachsene Zebrafisch-Männchen

untersucht. Ausserdem wurde in einem weiteren Versuch mittels Microarray Analyse das Transkriptionsprofil des UV-Filters EHMC in ausgewachsenen Zebrafisch-Männchen untersucht.

Das Benzodiazepin-Derivat Diazepam

Durch die Exposition männlicher Zebrafische mit 235 ng/L und 291 µg/L Diazepam wurde die Expression von 51 bzw. 103 Genen signifikant verändert. Damit Gene funktionellen Gruppen zugeordnet und diese identifiziert werden konnten, wurde eine Gene Ontologie (GO) Analyse durchgeführt. Nach der Behandlung mit 235 ng/L Diazepam wurden 57 funktionelle Gruppen identifiziert während nach der Behandlung mit 291 µg/L 40 Gruppen bestimmt wurden. In den identifizierten Gruppen sind vor allem Gene relevant, welche zu rhythmischen Prozessen insbesondere zum zirkadianen Rhythmus gehören. Zusätzlich zur GO Analyse wurde mit Hilfe von MetaCore eine Pathway Analyse durchgeführt. Diese zeigte auf, dass der zirkadiane Rhythmus einer der wichtigsten Pathway ist, welcher signifikant verändert wurde.

Die erhaltenen Microarray Resultate wurden anschliessend mittels qRT-PCR verifiziert. Dazu wurden folgende Gene untersucht: *arntl2*, *cry2b*, *cry5*, *nr1d1*, *per1, arr3*, *gabrr1* und *hsd17β3.* Ausserdem wurde die Änderung dieser Gene auch in Zebrafisch Larven, welche mit 273 ng/L und 273 µg/L Diazepam exponiert wurden, untersucht.

Im Anschluss an diese Experimente wurden die Effekte auf Gen-Ebene mit physiologischen Effekten wie z.B. das Schwimmverhalten gekoppelt. So konnte gezeigt werden, dass sich Zebrafisch Larven nach einer Exposition mit 273 µg/L Diazepam viel aktiver als unbehandelte Larven bewegen. Zusätzlich zum Schwimmverhalten wurden die Hoden der Männchen auf Veränderungen hinsichtlich der verschiedenen Entwicklungsstadien der Spermien histologisch untersucht. Es traten keine signifikanten Änderungen der Entwicklungsstadien auf, jedoch wurde eine Veränderung des interstitiellen Gewebes beobachtet. Diese Studie führte zum Fazit, dass die durch Diazepam verursachten Genexpressionsänderungen mit neurologischem Verhalten gekoppelt werden können. Dies ist aber nur für hohe und nicht für tiefe umweltrelevante Konzentrationen möglich.

Der Proteinkinase Inhibitor PKC412

Die Exposition von Zebrafisch Larven mit 1.3 und 21 µg/L PKC412 führte zu einer signifikanten Änderung von 259 bzw. 511 Genen. Angiogenese, Apoptose, Antwort auf DNA Schäden und Antwort auf oxidativen Stress sind einige der durch PKC412 veränderten Pathways. Wiederum wurden Verhaltens-, Entwicklungs- und physiologische Effekte bei

diesen exponierten Tieren untersucht, um eine Korrelation zwischen den Genexpressionsänderungen und Effekten in der Entwicklung und Physiologie zu ermöglichen. Ausserdem wurden die Genexpressionänderungen des zirkadianen Rhythmus vertiefend untersucht. Hierbei wurden jedoch keine Effekte auf das Schwimmverhalten beobachtet. Zusätzlich wurden mögliche Effekte durch PKC412 auf die Angiogenese mittels doppel transgenen Zebrafisch-Embryonen (Tg(fli1a:EGFP)y1; Tg(gata1:dsRed)sd2) untersucht. Es konnten keine signifikanten Effekte bei der Blutgefässbildung beobachtet werden. Apoptotische Zellen wurden hauptsächlich bei den olfaktorischen Plakoden nach einer Exposition mit 31 µg/L PKC412 gefunden. Im Gegensatz dazu, traten DNA Schäden bei beiden Konzentrationen (1.6 µg/L und 31 µg/L) von PKC412 auf. Es wurden jedoch nicht vermehrt reaktive Sauerstoffspezies gebildet. Fazit dieser Studie ist, dass Genexpressionsänderungen, welche durch PKC412 ausgelöst wurden, nur eingeschränkt mit physiologischen Effekten gekoppelt werden können. Diese Aussage trifft vor allem für die hohen PKC412 Konzentrationen zu. Mit Ausnahme der DNA Schäden war es anderseits nicht möglich die Genexpressionsänderungen der tiefen Konzentration von PKC412 mit physiologischen Effekten zu koppeln.

UV-Filter EHMC

Adulte Zebrafisch Männchen wurden mit 2.2 µg/L und 890 µg/L EHMC (nominale Konzentrationen) exponiert. Dies führte zu einer Expressionsänderung von 1096 bzw. 1137 verschiedenen Genen. 708 der signifikant veränderten Gene wurden bei beiden Konzentrationen gefunden, wobei diese Gene auch immer in der gleichen Richtung reguliert waren. Eine Gen Ontology Analyse wurde durchgeführt, um funktionelle Gruppen von Genen zu identifizieren. Bei beiden EHMC Konzentrationen wurden als Resultat dieser Analyse 1637 verschiedene Kategorien erhalten. Unter diesen Kategorien wurden vor allem zelluläre Prozesse, Entwicklungsprozesse und Antworten auf verschiedene Stimuli (endogene wie hormonelle) gefunden. Zusätzlich zu dieser Analyse wurde eine Pathway Analyse mittels MetaCore durchgeführt. 33 Mappen (33 bei 2.2 µg/L und 36 bei 890 µg/L EHMC) waren bei beiden Konzentrationen dieselben. Die wichtigsten Mappen sind: Gewebe Umgestaltungen, Wundheilung, Antworten auf DNA Schäden und Apoptose. Fünf verschiedene Gene (*vtg1*, *ptgds, igfbp1, rbp2a* und *gadd45b)* wurden mittels qRT-PCR in den Ganzkörperproben und zusätzlich in Gehirn, Leber und Hoden untersucht, um die erhaltenen Expressionsänderungen zu verifizieren. Um die Effekte von EHMC auf die hormonellen Pathways zu untersuchen, wurden 7 zusätzliche Gene (*vtg3*, *esr1*, *esr2b*, *ar*, *hsd17β3*, *cyp19b* und *cyp19a*) mittels qRT-PCR untersucht. Es wurden unterschiedliche Trends in der Genexpressionsänderungen der Ganzkörperproben im Vergleich zu den verschiedenen Geweben beobachtet. Man kann daher sagen, dass sich die Genexpressionen in den

verschiednen Geweben unterscheiden und oft nicht vergleichbar mit Ganzkörper-Antworten sind.

Aus diesem Grund war es nicht möglich, eine spezifische Wirkungsweise von EHMC aufzuzeigen. EHMC kann demnach unterschiedliche hormonelle Effekte in den verschiedenen Organen bzw. im ganzen Körper auslösen. Damit ein kompletteres toxikologisches Profil von EHMC skizziert werden kann, müssten zusätzlich physiologische Effekte und Effekte auf die Fortpflanzungsfähigkeit und Reproduktion untersucht werden.

Die erhaltenen Daten der *in vivo* Experimente zeigen, dass mittels einer Microarray Analyse neue Erkenntnisse über die Wirkungsweise der untersuchten Substanzen generiert werden. Genexpressionsänderungen sind zumeist sehr sensitiv und treten deshalb oft schon bei tiefen, nahe umweltrelevanten Konzentrationen auf (Diazepam, EHMC). Korrelationen zu Änderungen im Schwimmverhalten (Diazepam, PKC412) oder zu unterschiedlichen zellulären Prozessen (Angiogenese, Apoptose, Antwort auf DNA Schäden und Bildung von reaktiven Sauerstoffspezies; PKC412) treten jedoch meist erst bei Konzentrationen auf, welche bis zu 10'000 mal höher sind als durchschnittliche Umweltkonzentrationen der besagten Stoffe.

Zusammenfassend zeigt diese Arbeit ein neueres und detailierteres Bild über die Wirkungsweise von Diazepam, PKC412 und EHMC auf. Toxicogenomics war in diesem Zusammenhang eine angemessene Methode, um viele Informationen über Änderungen im Transkriptom zu erhalten. Nichtsdestotrotz, war es nur teilweise möglich die erhaltenen Genexpressionsänderungen mit phyisologischen Veränderungen zu verknüpfen. Aus diesem Grund sollten künftig vermehrt Anstrengungen unternommen werden, um diese Zusammenhänge besser aufzeigen zu können. Zusätzlich muss der Wert von Toxicogenomics für Umweltrisikoanalysen noch genauer untersucht werden.

Chapter 1

General Introduction:

Human pharmaceuticals and personal care products in the environment

1.1 Pharmaceuticals and personal care products in the environment

In the last decade, steadily increasing data on levels of environmental residues of pharmaceuticals and personal care products (PPCPs) have been reported. As a result, multiple research efforts have been initiated to scrutinize possible input pathways and fate of these newly emerging pollutants.

PPCPs constitute a group of a wide number of largely consumed compounds including therapeutic drugs, veterinary drugs, diagnostic agents, fragrances, cosmetics, sunscreen products, and nutraceuticals (e.g. vitamins). They are manufactured in large amounts as outlined in the following chapters. If they are easily broken down, processed by the human body or degraded quickly, only small concentrations of the parent compound occur in the environment independent of their production volume. However, PPCPs are still of concern as they may be active at extremely low concentrations, they are widespread and continuously released, and may have unpredictable biochemical interactions in mixtures or could biomagnify in the food chain, and therefore affect aquatic organisms. Some of the known effects are delayed development in fish (e.g. Raldúa et al., 2008) or even disruption of fish populations (Kidd et al., 2007) and delayed metamorphosis in frogs (Veldhoen et al., 2006), to name a few of the implications.

The following chapters focus on the input pathways of PPCPs into the aquatic environment, their occurrence and their potential effects on humans and the environment.

1.1.1 Exposure routes

PPCPs including their metabolites and transformation products can be discharged into the environment through different exposure routes. PPCPs mainly enter the sewage treatment plants (STPs) as components of domestic, hospital and industrial waste (Figure 1) including excretion by humans and animals, leaching from landfills, disposal of expired and unused PPCPs in the toilet, release of unabsorbed externally applied PPCPs (e.g. sun-screen) to surface waters and use of sewage solids for soil amendment and fertilisation. Once they have entered STPs, PPCPs can be removed by several routes (Fent et al., 2006; Suárez et al., 2008; Monteiro and Boxall, 2010). These removal mechanisms include sorption to solids, volatilization and biological and chemical transformation. Depending on their sorption coefficient (K_d, which includes absorption and adsorption), sorption to sludge can occur (Daughton and Ternes, 1999; Joss et al., 2005; Suárez et al., 2008). Volatilization is negligible for pharmaceuticals due to their physical properties. However, it is an important

process for instance for the fragrance celestolide (Suárez et al., 2008), and also for other PPCPs. Additionally to sorption and volatilization, PPCPs can be biodegraded into lower molecular weight products. Complete mineralization, partial degradation or minor chemical modifications can occur (Daughton and Ternes, 1999; Joss et al., 2005). The extent of biological transformation may vary greatly from compound to compound. Several studies showed that at least 90 % of the analgesic ibuprofen undergoes biotransformation (Buser et al., 1999; Joss et al., 2005; Reif et al., 2008). In contrast, only 15 % of the antiepileptic drug carbamazepine is lost due to biotransformation (Joss et al., 2005; Reif et al., 2008; Suárez et al., 2008).

Despite treatment, residues of most commonly used PPCPs are still present in the effluents of STPs, since many of these polar compounds are often incompletely removed due to their remarkable persistence; e.g. carbamazepine, which is neither sorbed nor biotransformed (Suárez et al., 2008). The treated wastewater is then mostly discharged into surface waters or sometimes irrigated onto fields. Additionally, primary and secondary sludge containing sorbed PPCPs is deposited on land. It has been reported that PPCPs contained in sewage sludge can be leached out, infiltrate into the groundwater (Khan and Ongerth, 2002; Kupper et al., 2004; Kinney et al., 2006), and subsequently into drinking water. In addition to consumption of contaminated drinking water, there are also other indirect (direct ones are drugs, sunscreens, cosmetics etc.) routes of exposure for humans; e.g. consumption of fish containing PPCPs, and dermal contact during showering with domestic water as well as swimming in PPCPs contaminated surface water.

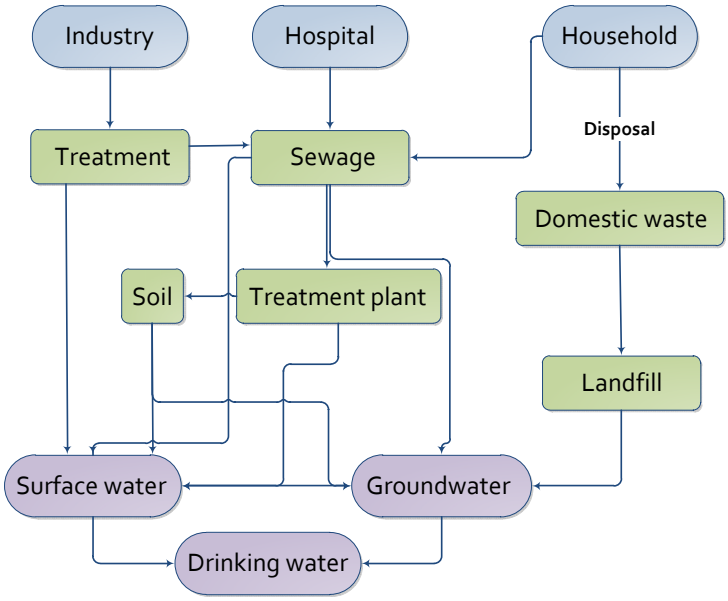


Figure 1

Exposure routes of human pharmaceuticals and personal care products into wastewaters and aquatic environment (adapted from Ternes, 2006)

1.1.2 Occurrence of PPCPs in the aquatic environment

PPCPs in STPs have been increasing over the last decade due to their growing number, production volumes, and extended use. Furthermore, in recent years lowered limits of detection in high performance liquid chromatography-tandem mass spectrometry and gas chromatography-tandem mass spectrometry have led to more comprehensive knowledge of residues of different pollutants in all kind of liquid media (wastewater, surface water, ground water, drinking water) and solid matrices (sewage sludge, soil, sediment) in the ng/L to low µg/L range (Figure 2) (Daughton and Ternes, 1999; Fent et al., 2006; Kümmerer, 2008; Suárez et al., 2008; Monteiro and Boxall, 2010). However, even these low concentrations remain a cause for concern since some of these compounds are biologically active at low concentrations, and generally effects on non-target species are mainly unknown.

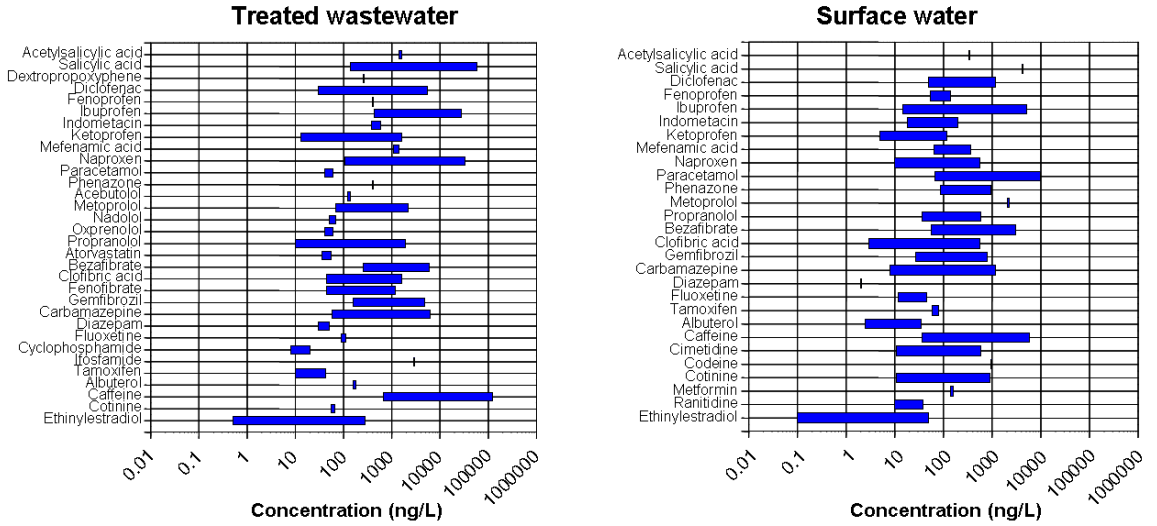


Figure 2

Concentration ranges of pharmaceuticals in (a) treated wastewater and (b) surface water. According to Fent et al., 2006

One of the most common classes of pharmaceuticals, a major category of PPCPs, detected in the environment are lipid regulators, particularly fibrates. Bezafibrate, gemfibrozil and fenofibrate have been detected at µg/L ranges in treated wastewater (Figure 2) (Fent et al., 2006). Clofibric acid is commonly reported in surface water and STPs effluents in the µg/L range and has even been detected in drinking water (Heberer and Stan, 1997). Beta blockers, too, are among the most prescribed drugs, so they are also frequently detected in STPs effluents and surface waters, where they occur in the range of ng/L to µg/L (Figure 2) (Fent et al., 2006).

Furthermore, there are many other pharmaceuticals occurring in the effluents of STPs (Figure 2). Several pharmaceuticals such as diclofenac, sulfamethoxazole and carbamazepine are only partially removed in STPs (Joss et al., 2006). Carbamazepine belongs to the most often used antiepileptics and has been frequently detected in STP effluents and surface waters. In STP effluents, concentration above 1 µg/L were detected, whereas in surface waters the concentrations were mostly in the ng/L range (Figure 2) (Fent et al., 2006). This brief overview and the few examples show that surface waters can be contaminated by many different PPCPs.

1.1.3 Ecotoxicological effects on non-target species

Adverse effects on aquatic organisms can occur as PPCPs and potentially also their metabolites are biologically active. Drugs as a major part of PPCPs are mostly designed to interact with cellular receptors at low concentrations and they elicit specific biological effects. The effects of drugs on lower vertebrates could be similar to those occurring in mammals, since many target receptors have been conserved during evolution (Fent et al., 2006; Owen et al., 2007; Gunnarsson et al., 2008; Christen et al., 2010). However, there are also PPCPs such as cosmetic additives which have unexpected effects on lower organisms, due to their differences in targets and metabolism (Daughton and Ternes, 1999).

Aquatic organisms are continuously exposed to PPCPs via wastewater at contaminated sites and the uptake includes branchial (gills), dermal (skin) and oral (gastrointestinal tract) absorption (Kennedy et al., 1995). PPCPs which come into contact with gills mostly penetrate the epithelium through the cells (transcellularly), and very rarely through tight junctions (paracellularly) (Kennedy et al., 1995).Thus far, there is no evidence of active transport through the gills into the blood. Therefore, it is assumed that passive diffusion is the main transport mechanism. In addition to the gills, the gut epithelium is an important uptake route for PPCPs. There, PPCPs can enter via the same routes as food or water uptake. The overall importance of uptake by the skin is unknown. There are studies which showed that cutaneous absorption is more important in small fish due to larger ratio between skin surface area to gill surface area (Lien and McKim, 1993). However, the uptake is also dependent on external physical factors such as dissolved oxygen, water pH and temperature (Kennedy et al., 1995). Due to this continuous exposure, multi-generation exposure can occur and depending on body size, low-dose effects are also particularly relevant.

In addition, PPCPs are often present in the environment as complex mixtures which could result in additive, synergistic or antagonistic effects (Richards et al., 2004). Many polar

PPCPs show "pseudopersistence" in the environment, as they are continuously discharged via wastewater. This will lead to accumulation of PPCPs in aquatic organisms and potentially to biomagnification in the food chain as shown for antidepressants (Brooks et al., 2005). Furthermore, there are also PPCPs having endocrine activity, such as synthetic oestrogens (Länge et al., 2001) or progestins (Zeilinger et al., 2009; Paulos et al., 2010) commonly used in contraceptives. Several studies have shown that reproduction of aquatic organisms is affected at even low concentrations of synthetic oestrogens. Observed effects were changes in the sperm density, gonad size, reduced viability of eggs and male sex reversal (Länge et al., 2001; Zillioux et al., 2001; Schultz et al., 2003; Thorpe et al., 2003; Hogan et al., 2010).
However, even up to now only little data are available for evaluating potential risks to the aquatic environment, except for some endocrine disruptors. As PPCPs remain a challenging problem for both humans and aquatic organisms, further effort should be put into ecotoxicological research of PPCPs.

1.1.4 Biotransformation in aquatic organisms

Aquatic organisms are continuously exposed to a number of PPCPs. Due to this, organisms have to avoid compound accumulation in their body. An efficient protection via metabolism, elimination and adaptation is therefore essential (Livingstone, 1998; Van Leeuwen and Vermeire, 2007). There are two classes of elimination reactions, phase-I and phase-II reactions (Figure 3). Phase-I reactions usually precede phase-II reactions, though not necessarily. During these processes, polar groups are either introduced or unmasked resulting in more polar metabolites of parent compounds. In the case of pharmaceuticals, phase-I reactions can lead to inactivation but also to activation of a compound generating biologically more active or toxic metabolites (Halling-Sorensen, 1998). Phase-I reactions may occur by oxidation, reduction and hydrolysis (Livingstone, 1998). Oxidation involves the enzymatic addition of oxygen or removal of hydrogen, carried out by cytochrome P450-dependent monooxygenases (CYP), most often in the liver, the flavin-containing monooxygenases (FMO) and the peroxidases (Guengerich, 1992). Oxidative reactions (e.g. hydroxylation, N-oxidation, deamination, N-dealkylation) typically involve a cytochrome P450 hemeprotein, NADPH and oxygen. CYPs play a critical role in catalyzing reactions e.g. biosynthesis of steroid hormones, metabolism of xenobiotics to reactive metabolites, oxidation of unsaturated fatty acids etc. FMOs catalyze numerous monooxygenase reactions using NADPH. FMOs play an important role in the toxicity of various heteroatom-containing substances (Schlenk, 1998). In addition, peroxidases are also involved in oxidation processes. The peroxidase action involves the use of hydroperoxide as a substrate resulting in an oxidation of this substrate (Guengerich, 1992). Examples for pharmaceuticals metabolised via oxidative reactions are diazepam (N-dealkylation, deamination), ibuprofen

(aliphatic hydroxylation), acetaminophen (N-oxidation) and many more (Monteiro and Boxall, 2010). Reduction is also an important phase-I reaction, and PPCPs containing functional groups such as aldehydes, ketones, and alcohols are reduced through enzymatic reactions catalyzed by reductases and different dehydrogenases (Kennedy et al., 1995). Hydrolytic reactions can also occur in fish. PPCPs containing esters such as MS-222, amides and epoxides mostly undergo such reactions (Kennedy et al., 1995). Aspirin, clofibric acid, cocaine, lidocaine etc. were metabolised via hydrolysis reactions (Monteiro and Boxall, 2010).

If metabolites of phase-I reactions are sufficiently polar, they may be readily excreted at this point. However, many phase-I products are not rapidly eliminated and undergo a subsequent reaction, which involves conjugation of the compound with either sugars (glucuronidation) or peptides to form a more polar conjugate (Daughton and Ternes, 1999; Suárez et al., 2008). During phase-II reactions functional groups are linked to polar, negatively charged endogenous molecules. Important phase-II reactions are glucuronidation, sulphonation, methylation, acetylation and conjugation with amino acids and glutathione. Glucuronidation involves the conjugation of UDP-glucuronic acid to PPCPs. Acetaminophen, oxazepam (a metabolite of diazepam) and morphine are metabolised by glucuronidation (Monteiro and Boxall, 2010). During sulphonation reactions, sulphate is transferred to PPCPs containing phenolic groups as for instance in the metabolism of sulphonamides (Monteiro and Boxall, 2010). Biologic methylation is an important process in the metabolism of norepinephrine, serotonin and histamine. In addition, conjugation of PPCPs to various amino acids such as taurine and glycine can occur in fish (Kennedy et al., 1995). Glutathione S-transferases (GST) catalyze the formation of N-acetylcysteine derivatives of PPCPs resulting in metabolites, which were generally less toxic and more water soluble (Kennedy et al., 1995). The purpose of these reactions is to convert lipophilic chemical compound into more easily excreted polar products.

However, there are also PPCPs such as tetracyclines, penicillins, fluoroquinolones and β-blockers (with the exception of propanolol and betaxolol) which are excreted unchanged, whereas analgesics and anti-inflammatory drugs are extensively metabolised (Monteiro and Boxall, 2010).

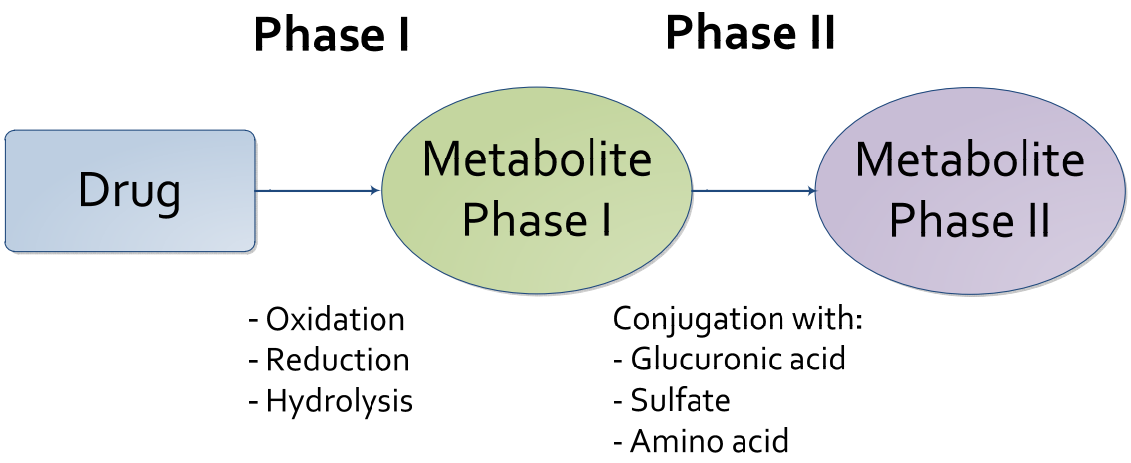


Figure 3

Metabolic approach to increase the polarity and excretability of drugs. (Adapted from Daughton and Ternes,1999)

In addition to phase I and II detoxification processes, there are also "phase 0 and III" processes found to occur in fish (Sturm et al., 2005). Proteins belonging to "phase 0" act as a cellular first line of defence by preventing the up-take of compounds by the cell (Glavinas et al., 2004; Xia et al., 2007; Szakacs et al., 2008). Multidrug resistance proteins (ABC transporters) are of high significance in this process. They form a barrier against drug, metabolites and toxicant up-take, and play an important role in distribution and excretion of a wide variety of compounds (Brinkmann and Eichelbaum, 2001). In addition, they are responsible for the efflux of metabolites, and are therefore, referred to the "phase III" detoxification system (Xu et al., 2005; Szakacs et al., 2008; Zaja et al., 2008; Loncar et al., 2010). However, the action of this detoxification system can be inhibited by certain substances such as verapamil that directly binds to the active site of the transporter (Caminada et al., 2008; Zaja et al., 2008). This can increase the toxicity of a number of drugs as they can not be readily removed from the exposed organism and therefore the exposure time is increased.

1.1.4.1 CYPA3

During the evolution of eukaryotic cells, mechanisms have emerged serving to protect animals and also plants against chemicals. The cytochrome family enzyme group (CYPs) is a part of this protective mechanism system, representing an important class of phase-I enzymes. CYPs are intracellular type III membrane proteins which activate molecular oxygen to metabolize a great variety of organic chemicals. They can be found in most organisms, organs and tissues such as liver, kidney, breast, prostate, skin, gonads, placenta, brain, lung, spleen, pancreas and gastro-intestinal tract. In fish, the site of the major expression is the liver and kidney (Stegeman, 1989; Buhler and Wang-Buhler, 1998).

In our research (Chapter 2) we focus on the isoenzyme CYP3A. The CYP3A family forms the largest subfamily of CYPs found in the liver and small intestine (Hegelund and Celander, 2003; Lee and Buhler, 2003; Nallani et al., 2004), and plays an important role in the metabolism of endogenous substances and xenobiotics. Several drug-drug interactions are associated with the induction of this enzyme (Tseng et al., 2005). CYP3A is not only common to mammals, but it can also be found in several fish species; e.g. rainbow trout CYP3A27 and CYP3A45 (Lee et al., 1993; Lee and Buhler, 2003), medaka CYP3A38 (Kullman et al., 2000) and CYP3A40 (Kullman and Hinton, 2001), killifish CYP3A30 and CYP3A56 (McArthur et al., 2003), *Danio rerio* CYP3A65 (Tseng et al., 2005) and fathead minnow CYP3A126 (Christen et al., 2010b).

There are remarkable species differences in the response of CYP3A to xenobiotics (Roymans et al., 2004). Rifampicin for example is a strong inducer of the human CYP3A4, but not in mice, whereas pregnenolone 16α-carbonitrile (PCN) is a strong activator of the murine CYP3A, but not in humans (Vignati et al., 2004). Zebrafish seem to elicit the same response as in mice. It was shown that PCN is also an inducer of CYP3A65 in zebrafish liver (Bresolin et al., 2005). In the same study it was shown that nifedipine and clotrimazole were not able to induce CYP3A65 *in vivo*. However, these compounds induced CYP3A *in vitro* (Moore et al., 2000). Additionally, rifampicin and dexamethasone, which are known human CYP3A inducers, also induce CYP3A activity in grass carp (Li et al., 2008). However, in zebrafish only rifampicin is a CYP3A inducer, whereas dexamethasone is an inhibitor (Tseng et al., 2005). In carp, CYP3A activity was significantly inhibited by antidepressant drugs such as paroxetine, fluvoxamine, and fluoxetine (Thibaut et al., 2006). These compounds are also well-known inhibitors of human CYP3A4 (Tredger and Stoll, 2002). In addition, significant inhibition of carp CYP3A was shown for gemfibrozil, clofibrate and diclofenac (Thibaut et al., 2006). The antifungal agents ketoconazole, miconazole, and clotrimazole, and EE2 inhibited CYP3A-catalyzed activities in different fish species (Miranda et al., 1998; Hegelund et al., 2004; Hasselberg et al., 2005). Typical mammalian inducers are not able to induce CYP3A in the fish cell line PLHC-1 (Celander et al., 1996). Due to this fact, extrapolation from mammals to other vertebrates such as fish is not always possible in this respect.

1.2 Pharmaceuticals

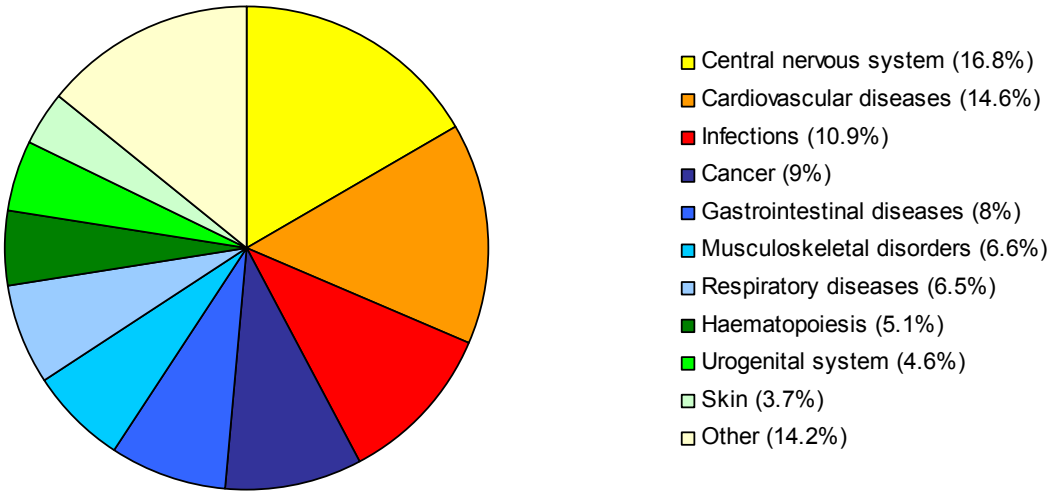
The high consumption of pharmaceuticals, especially in industrialized countries, has led to contamination in the environment. There are more than 4000 pharmaceuticals with different physico-chemical and biological properties and different modes of action (Beausse, 2004). It was estimated that the annual global consumption of drugs by humans is around 100,000 tons (Ternes, 2006). In Europe approximately 3000 different pharmaceuticals are commonly

used. Among them are painkillers, antibiotics, antidiabetics, beta blockers, contraceptives, lipid regulators, antidepressants, neuroreactive drugs and cytostatic agents. In 2008, most pharmaceuticals consumed in Switzerland belonged to the group of neuro-active compounds (16.8%) and around 9% of totally consumed pharmaceuticals were cancer drugs (Figure 4).
As a consequence, a mixture of pharmaceuticals and their metabolites will enter the STPs. Depending on their water solubility, polarity and persistence, pharmaceuticals may not be completely eliminated or transformed, and occur in the aquatic environment (Corcoran et al., 2010). More than 100 different pharmaceuticals have been reported in the high ng/L and low µg/L range in STP effluents (Halling-Sorensen, 1998; Daughton and Ternes, 1999; Fent et al., 2006). In surface waters, pharmaceuticals rarely exceed concentrations of 100 ng/L (Trudeau et al., 2005). In addition, certain pharmaceuticals were detected in the low ng/L range in oceans (Weigel et al., 2004), groundwater (Corcoran et al., 2010) and even in drinking water (Bruce et al., 2010). Although these concentrations are low, pharmaceuticals may pose a risk to aquatic organisms as they were designed to elicit specific biological effects at low concentrations. As aquatic organisms are continuously exposed to pharmaceuticals, there is a risk for uptake via dermal and gill surfaces, orally through diet, or maternally via lipid reserve of eggs (Erickson et al., 2008). Therefore, there is a potential of unintended side effects on non-target organisms in the aquatic and terrestrial environment (Daughton and Ternes, 1999; Fent et al., 2006; Monteiro and Boxall, 2010).

Several pharmaceuticals are known to induce such effects. A prominent example is the synthetic oestrogen 17α-ethynylestradiol (EE2), which is extremely potent in fish. It was shown to induce feminisation in fish at environmentally relevant concentrations, including induction of vitellogenin in males (Scholz et al., 2004), formation of female reproductive duct in testis (Jobling et al., 1998), and induction of inter-sex (Jobling et al., 1998; Länge et al., 2001; Shved et al., 2008). Additionally to this drug class, there are nonsteroidal anti-inflammatory drugs (NSAIDS), which are known to affect reproduction in fish. For instance indomethacin disrupts the process of oocyte maturation and ovulation in zebrafish (Lister and Van Der Kraak, 2008), and ibuprofen is able to alter the spawning pattern in medaka (Flippin et al., 2007). Furthermore, lipid lowering agents may also interfere with fertility (Runnalls et al., 2007). Although various effects of pharmaceuticals have been documented, evidence of these effects on the biochemical and molecular level, as well as on populations is as yet missing. However, the decline of vulture populations in the Indian subcontinent is the result of the exposure to the anti-inflammatory drug diclofenac (Oaks et al., 2004).

In addition to these effects, bioaccumulation and persistence of pharmaceuticals is also of concern (Corcoran et al., 2010). Furthermore, pharmaceuticals are often released as

complex mixtures in the environment. The combined effects of these are largely unknown (Tixier et al., 2003; Stackelberg et al., 2004), but additive interactions are demonstrated *in vitro* (Fent et al., 2006). Addressing the lack of knowledge concerning the effects of



pharmaceuticals and the ecotoxicological risks associated with their environmental occurrence, this thesis focuses on selected pharmaceuticals.

Figure 4

Distribution of pharmaceuticals on the Swiss market in 2008. The main group used in Switzerland belongs to pharmaceuticals against diseases in the central nervous system followed by pharmaceuticals against cardiovascular diseases (source: IMS Health GmbH, Hergiswil).

Our studies mainly focus on two different pharmaceuticals belonging to different compound classes. In a first study (Chapter 3), the effects of diazepam on the gene expression pattern of adult zebrafish and zebrafish eleuthero-embryo were investigated. This neuro-active compound was chosen because of its high usage, its occurrence in the environment and the need for more complete effect data for its risk assessment (Straub, 2008). Although diazepam is an "old" compound (admission to the market in 1963), there is still the need for more knowledge about its effects on non-target species such as fish.

In a second study (Chapter 4), the effects of a novel antineoplastic drug, midostaurin (PKC412), on the gene expression pattern of zebrafish eleuthero-embryos were investigated. Additionally, we searched for correlations between altered gene expressions and effects on the physiological level. This anti-cancer agent that will be on the market only in the future was chosen because of its high biological activity and unknown environmental risks. The ecotoxicological analysis prior to its introduction into the environment allows a prospective risk analysis.

1.2.1 γ-Aminobutyric acid (GABA) signalling: Diazepam

Diazepam is the active ingredient of Valium® and belongs to the benzodiazepine group, which has a widespread application in human medicine (Jouvel et al., 2000). It has anxiolytic, sedative, muscle-relaxant, anti-convulsive and anti-epileptic properties (Shader and Greenblatt, 1977). Due to its high use and incomplete degradation, concentrations of up to 0.66 µg/L (van der Hoeven, 2004) were detected in effluents of sewage treatment plants. In surface water, concentrations ranged from 0.04 µg/L in Germany (Ternes, 1998) to 0.66 µg/L in Belgium (van der Ven et al., 2004). Acute toxicity and additional data are reported (Straub, 2008). However, it remains unknown whether environmental exposures lead to negative effects on aquatic life. Therefore, there is a need to analyze for effects at more environmentally realistic concentrations, which is done in this thesis.

Benzodiazepines act through binding to the $GABA_A$ receptor resulting in central nervous system (CNS) depression. The $GABA_A$ receptor consists of five subunits (Carr and Chambers, 2001). Binding of benzodiazepines, barbiturates and neuroactive steroids to the GABA receptor potentiates GABA action, which results in depression of the CNS. As in mammals, three subtypes of GABA target receptors ($GABA_A$, $GABA_B$ and $GABA_C$) occur in fish, and the binding site for benzodiazepines in the brain is suggested to be highly conserved between fish and mammals (Carr and Chambers, 2001). In non-target species acute diazepam toxicity lies in the high mg/L range, whereas chronic toxicity is in the low mg/L range (Straub, 2008). The measured "no observed effect concentration" (NOEC) of diazepam in early life stage tests with *D. rerio* was found to be 273 µg/L, and the "lowest observed effect concentration" (LOEC) was 2.57 mg/L (Straub, 2008). Exposure of fish to diazepam may impair locomotion (Zhdanova et al., 2001; Nunes et al., 2008), affect vision (Salas et al., 1992) and can produce anxiolytic effects at concentrations ranging from 1.25 to 5 mg/L, doses that do not cause sedation in fish (Bencan et al., 2009). Nevertheless, there is a lack of chronic environmental toxicity studies with focus on the modes of action. Furthermore, more specific effects should be investigated as a proper ecological risk assessment of diazepam at environmentally realistic concentrations in aquatic organisms is missing.

1.2.2 Protein kinase C inhibition: Midostaurin (PKC412)

Midostaurin (PKC412) is a member of the family of indolocarbazoles and a selective inhibitor of several isoforms of protein kinase C (PKC) in humans. PKC412 has a broad kinase inhibition spectrum and is therefore not very selective, even at high affinity interaction (Kd < 100 nM; (Karaman et al., 2008). It is a sugar ring variant of staurosporine, which was

originally isolated from *Streptomyces staurosporeus* (Takahashi et al., 1989). PKC412 is able to inhibit a large variety of tyrosine kinases including FLT3 (FMS-like tyrosine kinase), PDGF (platelet derived growth factor) receptors and c-kit (stem cell factor) receptor (Fabbro et al., 2000). PKC412 has been developed as a therapeutic agent against acute myeloid leukaemia (AML), because of its ability to inhibit growth, angiogenesis and P-glycoprotein mediated multidrug resistance in tumour cells. In addition, PKC412 also affects other cellular processes such as immune responses or neuronal functions, such as human T-cell activation, proliferation and TNFα production (Si et al., 2005).

Since Midostaurin is in clinical phase III and not yet on the market, there are very few data available about its toxicity in aquatic organisms. In non-target species the acute and the chronic PKC412 toxicity lies in the lower µg/L range; exposure of zebrafish larvae to 100 nM (57 µg/L) PKC412 has resulted in a curved body axis (Chan et al., 2002). The measured "no observed effect concentration" (NOEC) of PKC412 in early life stage tests with *D. rerio* was 14 µg/L, and the "lowest observed effect concentration" (LOEC) was 43 µg/L (Novartis Pharma AG, internal data). The 96h-LC_{50} in *D. rerio* was 25 µg/L and the NOEC was 19 µg/L (Novartis Pharma AG, internal data). The predicted environmental concentration (PEC) is estimated in the range of 1.5 µg/L (Novartis Pharma AG, internal data).

Nevertheless, there is a lack of chronic environmental toxicity studies with focus on the modes of action on this pharmaceutical. Furthermore, ecological risk assessments of PKC412 at environmentally realistic concentrations in aquatic organisms are lacking.

1.3 Personal care products

Personal care products (PCPs) are substances mainly used to maintain hygiene and general-well being. They include fragrances (i.e. synthetic musks), cosmetics (i.e. skin-care cream, lotions, powders, perfumes, lipsticks, nail polish, make-up, hair dyes, hair sprays and gel, shampoos deodorants, etc.) sunscreens (UV-filters) and protective products such as insect repellents (DEET) and antimicrobials (e.g. triclosan).

There is a high consumption of PCPs in our daily life; e.g in 2000, the annual use of the synthetic musk galaxolide (HHCB) was 1473 t in Europe (Berset et al., 2004). These products can be released directly into recreational waters or volatilized into the air (e.g. musks). Thus they were also found in the environment. HHCB was detected in water at concentrations up to 5 µg/L in Germany (Bester, 2005) and up 6.9 µg/L in Switzerland (Berset et al., 2004). In addition, ingredients of sunscreens were detected at high levels in the aquatic environment (Balmer et al., 2005; Zwiener et al., 2007; Rodil and Moeder, 2008).

As a result, aquatic organisms are continuously exposed to them and possibly negatively affected. It was shown that commonly used fragrances such as tonalide (AHTN) and HHCB are not only oestrogenic (Seinen et al., 1999; Schreurs et al., 2002), but also elicit acute toxicity in some aquatic organisms at environmentally relevant concentrations (Breitholtz et al., 2003; Gooding et al., 2006). HC Orange No.1, a colour additive, was shown to bioaccumulate in goldfish (Sun et al., 2006). The effects of fragrances, in particular synthetic musks, and UV-filters, in particular EHMC, were described in detail in the following chapters (1.3.1 and 1.3.2).

1.3.1 Fragrances: Synthetic musks

Synthetic musks are widely used as inexpensive fragrances in personal care products. They are structurally similar chemicals which mimic the odour of the expensive, natural Asian musk deer and include synthetic nitro musks (e.g. musk ketone, musk xylene etc.) and synthetic polycyclic musks (indane and tetraline derivatives). These artificial musks do not occur naturally and there is no structural similarity to natural musk compounds. Nowadays, mostly polycyclic musks were used as nitro musks are known to be persistent and to induce adverse effects (Tas et al., 1997; Chou and Dietrich, 1999). Polycyclic musks are used in almost all consumer products, such as perfumes, cosmetics, soaps, shampoos, laundry detergents, fabric softeners, household cleaners, air fresheners, and other household products. Used in high amounts (tons per year), they are ubiquitous pollutants in aquatic environments and have received increasing attention (Rimkus and Wolf, 1996; Kafferlein et al., 1998). Although of low toxicity, their persistence and ability to bioaccumulate have raised concerns about them as environmental pollutants and also for human health.

Polycyclic musks such as HHCB and AHTN were found in fish in Italy (Draisci et al., 1998) and at different sampling sites in Germany (Winkler et al., 1998; Heberer et al., 1999). In addition, they were already detected in the 90s in the river Glatt in Switzerland (Muller et al., 1996) and also recently at different sampling sites in South Korea (Lee et al., 2010). HHCB and AHTN bioconcentrate in aquatic biota (Rimkus, 1999) and have been measured in human blood (Hutter et al., 2005), adipose tissue (Rimkus and Wolf, 1996), and breast milk (Reiner et al., 2007)). Due to the fact that synthetic musks are small compounds, they are also able to inhibit ABC transporters in aquatic organisms. Musk ketone, musk xylene, HHCB and AHTN were able to inhibit multixenobiotic resistance (MXR) transporter activity in the California mussel (*Mytilus californianus*) (Smital et al., 2004). As a consequence, synthetic musks may enhance the toxicity of other MXR substrates and the may accumulate in cells.

1.3.2 UV-filters: EHMC

UV-filters are organic or inorganic ingredients of personal care products whose purpose is to filter UV-A and/or UV-B radiation from sunlight in order to protect the human skin from their negative effects. UV-filters are increasingly used not only in sunscreen but also in other products used daily e.g. cosmetics, body lotion, hair spray, shampoos etc, as well (Balmer et al., 2005; Fent et al., 2010). As a result of their various applications, UV-filters may enter the aquatic environment either directly via wash-off from skin and clothes or via effluents of STPs or swimming pool waters, where rather high levels of different UV-filters were measured (Balmer et al., 2005; Zwiener et al., 2007; Rodil and Moeder, 2008). They enter the environment either directly or indirectly via wastewater. Further sources of UV-filter residues are landfill leachates as well as deposition from building components which are protected with coatings (Plagellat et al., 2006).

Many organic UV-filters are lipophilic and photostable. Therefore, they are relatively stable in the aquatic environment and maintain the potential of adsorbing into sewage sludge (Plagellat et al., 2006). Additionally, they bioaccumulate in fish (Buser et al., 2006; Fent et al., 2010) and in human breast milk (Schlumpf et al., 2008). Some UV-filters interfere with the sex-hormone system and act therefore as endocrine disruptors. Since significant amounts of these chemicals are used today, there are major concerns and needs for a better understanding of effects on fish and also of their mode of action. For some UV-filters hormonal activity was documented *in vitro* as well as *in vivo* (Schlumpf et al., 2001; Schlumpf et al., 2004; Gomez et al., 2005; Kunz and Fent, 2006b). Organic UV-filters demonstrated oestrogenic activity and adverse effects on fertility and reproduction in fish (Kunz et al., 2006a; Kunz et al., 2006b; Weisbrod et al., 2007). In previous studies 3-benzylidene camphor (3-BC), benzophenone-1 (BP-1) and BP-2 led to vitellogenin (Vtg) induction in juvenile fathead minnows (Kunz and Fent, 2006b) and benzophenone-3 (BP-3) in medaka (Coronado et al., 2008). Vtg induction has been reported for 3-BC in rainbow trout (Holbech et al., 2002), and for 4-methyl-benzylidene camphor (4-MBC) and 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) at high concentrations in male medaka (Inui et al., 2003).

In this thesis we are mainly interested in the UV-filter EHMC as it is one of the most widely used UV-filters in sunscreens (see Chapter 5 and 6). The environmental concentrations of EHMC range between 0.01-0.1 µg/L in treated wastewater and up to 19 µg/L in untreated municipal wastewater (Balmer et al., 2005). EHMC residues were detected in lakes and rivers (Balmer et al., 2005; Fent et al., 2010), and in coastal seawater up to 390 ng/L (Langford and Thomas, 2008). This compound was also found at levels of 0.26 to 5.61 µg/L

in raw drinking water (Loraine and Pettigrove, 2006), and unexpectedly, even at very remote environments such as the Pacific Ocean (Polynesia) (Goksoyr et al., 2009).

EHMC is lipophilic (logP = 5.66; Zenker et al., 2008) and accumulates in aquatic biota (Fent et al., 2010). It was found in different trophic levels and tends to bioconcentrate along the food-chain (Figure 5).

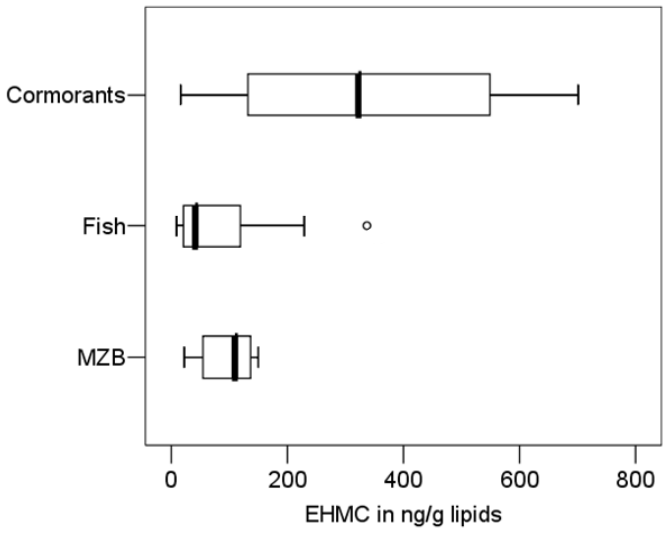


Figure 5

Box-whisker plots of UV-filters residues of EHMC aggregated for three trophic levels (cormorants, fish, MZB=macrozoobenthos). ○ = outlier (according to Fent et al., 2010)

Despite its widespread presence in the environment, little is known about the potential risk posed by EHMC to aquatic life. In recombinant yeast systems EHMC showed anti-oestrogenic and antiandrogenic with weak androgenic activity *in vitro* (Kunz and Fent, 2006a). Injection of high concentrations of EHMC in male medaka led to induction of Vtg (Inui et al., 2003), while no Vtg induction was observed in juvenile fathead minnows exposed to lower aqueous concentrations (Kunz and Fent, 2006a). These few studies are not conclusive and the potential oestrogenic activity of EHMC in fish needs further investigation. Moreover, the modes of action on the hormone system remain to be investigated.

1.4 Small fish models

Fish find frequent application as model organisms in aquatic toxicology. Small fish species commonly used in ecotoxicology are zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*).

1.4.1 Zebrafish (*Danio rerio*)

Danio rerio (Figure 6) is a small tropical fresh-water fish, which is native to rivers of northern India, northern Pakistan, Nepal and Bhutan. It not only is a common aquarium fish, but also an interesting model organism in biology. *Danio rerio* is the most popular model organism in embryonic development and often studied in (eco)toxicology (Raldua and Babin, 2007; Liedtke et al., 2008; Mehta et al., 2008).

Figure 6

Adult female zebrafish. The characteristic stripes running along the fins gave this species its name. Adult zebrafish are only about 3-5 cm in length. (Photo by S. Tinguely)

The unique features of *Danio rerio* are the short generation time, the large number of offspring and the transparency of embryos. Therefore, it is easy to conduct toxicological studies over the whole reproduction cycle. The basic body plan is already established in zebrafish embryos after 24 hours post fertilisation (hpf) and they hatch approximately after 2-3 days post fertilisation (dpf) (Figure 7).

At 5 dpf organogenesis of major organs is completed (Rubinstein, 2003). The transparency of the chorion eases the observation of different developmental stages (Kimmel et al., 1995). Zebrafish are already mature at the age of 3 months. Having such a short reproduction cycle, female zebrafish can lay more than a hundred eggs per day, even under laboratory conditions. This facilitates the use of zebrafish embryos for ecotoxicological research.

The sequencing of the zebrafish genome (http://zfin.org/) is highly promoted and places this freshwater fish in a privileged position for toxicogenomic studies. Due to genetic similarities to vertebrates, the zebrafish is also used as a model for human diseases and development, to name just a few fields.

Experiments with embryos are considered as alternatives to (adult) animal experiments due to animal welfare reasons. The embryo model is a complex, multicellular system, which

integrates interactions of various tissues and differentiation processes. This is an advantage over all other available systems.

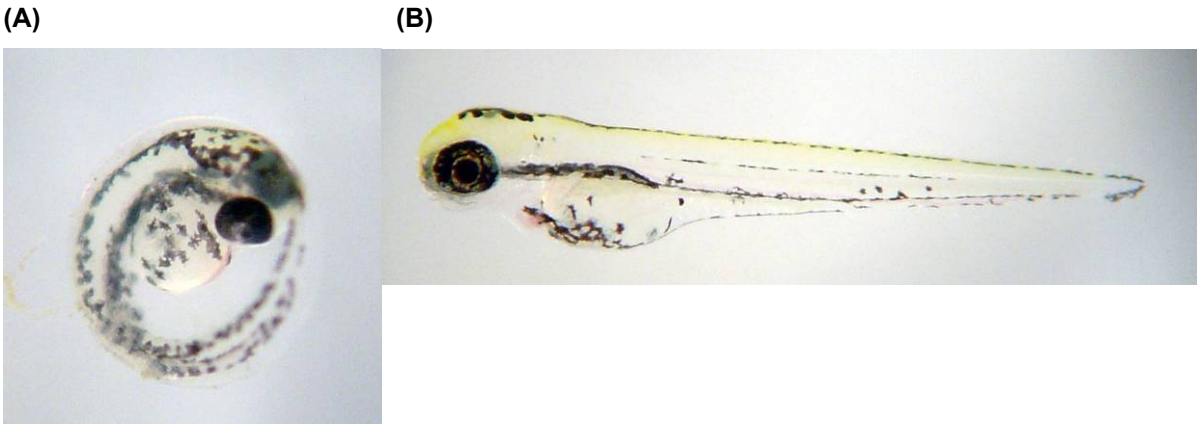


Figure 7

Zebrafish eleuthero-embryo. ***(A)*** *48 hpf.* ***(B)*** *72 hpf.*

A number of permanent cell lines from *Danio rerio* have been established. ZFL (Figure 8, liver cell line, (Ghosh et al., 1994), ZF4 cell line (24 hpf embryo), ZF13 and ZF 29 (fibroblast like cell line, (Collodi et al., 1992) and ZEM2 cell lines (from blastula stage embryo, (Ghosh and Collodi, 1994) are only some of the used cell lines for research. These laboratory cell lines are able to conserve some of their original *in vivo* tissue-specific gene expression, even though adapted to experimental culture (Ross et al., 2000). Due to the availability of such cell lines, it is possible to conduct large scale genomic analysis of drug-induced changes in mRNA expression levels of environmentally relevant pharmaceuticals in a hepatic *in vitro* system (Pomati et al., 2007).

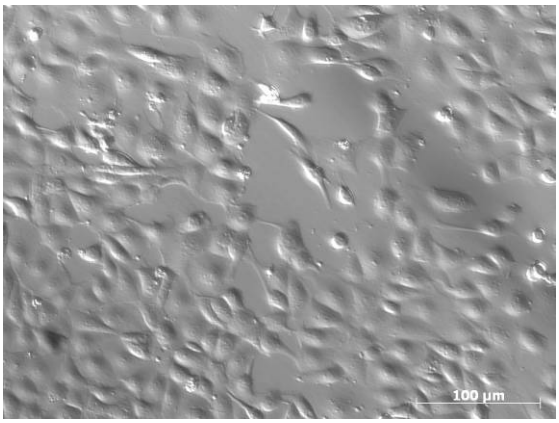


Figure 8

Cell morphology of zebrafish liver cell line (ZFL)

1.5 Toxicogenomics

Toxic effects of chemicals can lead to alterations of processes on molecular levels, e.g. gene expression, protein expression or metabolomic profiles. Up to now, standard toxicity tests provide little information about possible modes of action. The new field of toxicogenomics includes transcriptomics (gene expression), proteomics (protein expression) and metabolomics (metabolic profile). These novel methods allow a system-wide analysis of effects caused by chemical exposure. The advantage of toxicogenomics is that it uses the changes in gene and protein expression to detect subtle molecular-level changes in organisms that may precede toxic effects or propagate to higher level effects. Additionally, it can provide information about molecular changes in an organism before obvious manifestations of toxicity are present. Furthermore, microarrays allow studying of different endpoints in a single assay and it is possible to obtain characteristic patterns of gene expression that may allow identifying the toxic potential. As toxicogenomics has the potential to serve as a powerful tool to detect molecular effects and new biomarkers, we focus in this thesis on transcriptomics.

1.5.1 Transcriptomics

Thus far, Northern Blot, *in situ* hybridisation and quantitative RT-PCR allowed monitoring of only a small set of selected genes. In contrast, DNA microarray technologies allow screening for a large set of differentially expressed genes. Additionally, they can detect changes in gene expression before physiological changes e.g. behaviour, tissue damage, reproduction etc. can occur. Therefore, it is a tool for obtaining an early indication of a toxic response.

DNA microarrays basically comprise a glass slide where oligonucleotides were spotted (e.g. Agilent, Affymetrix). Depending on the brand, the oligonucleotides consist of 25 bases (Affymetrix) or 60 bases (Agilent). The oligonucleotides are either synthesized by photolithographic procedures (Affymetrix) or ink-jet principles as used by Agilent. Each gene is then represented by different oligonucleotide fragments, which are attached on the glass slide on a tiny section (Figure 9).

The principle behind microarrays is the hybridisation between the oligonucleotides on the chip and cRNA in the samples. The isolated mRNA is reversed transcribed into cDNA and then converted into double stranded cDNA. Thereafter, the cDNA is converted to cRNA using fluorescently labelled nucleotides. The labelled cRNA is then hybridised to the microarray. Unbound cRNA is washed off and the slides are scanned to obtain the appropriate signal intensities.

The use of cRNA instead of cDNA has two advantages. Firstly, RNA to DNA hybridisation has a higher sensitivity than DNA to DNA. Secondly, the isolated mRNA will be multiplied during the conversion from DNA to cRNA, which leads to the detection of signals that are based on a very low number of expressed mRNA.

Using Agilent microarrays there is also the possibility of performing one-colour or two-colour experiments. In one-colour experiments all probes are labelled with the same fluorescent dye and hybridised on separate slides. In contrast, performing two-colour experiments control and treated samples are labelled with different dyes and hybridised on the same slide. In this thesis we performed one-colour experiments using Agilent 4x44K zebrafish microarray.

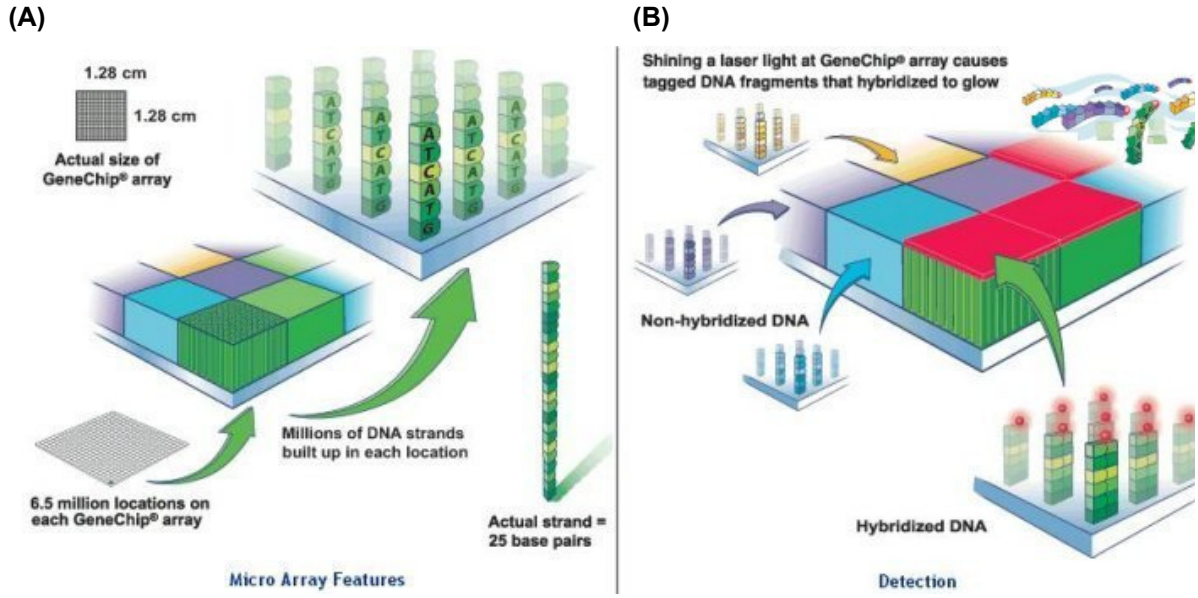


Figure 9

Basic construction of an Affymetrix chip. ***(A)*** *Features of a microarray. Each chip consists of 6.5 mio locations which were built up by millions of DNA strands. One DNA strand is 25 base pairs in length.* ***(B)*** *Hybridisation and detection (source: Affymetrix homepage http://www.affymetrix.com)*

Although this method allows the investigation of thousands of gene expression changes, there are some major concerns about the toxicological significance. From mRNA level to the protein level, several modification processes can take place, not always leading to changes in the protein expression. Therefore, effects on gene expression should always be correlated to effects on protein level, behavioural changes, physiological effects etc. to validate the toxicological relevance.

1.5.2 Ecotoxicogenomics

In response to exposure to PPCPs, changes in gene expression occur and altered patterns of mRNA expression can be analyzed by microarrays. Ecotoxicogenomics allows us to obtain a better understanding of toxicological mechanisms and to identify gene expression patterns that are representative of adverse outcomes (Neumann and Galvez, 2002; Snell et al., 2003; Coverdale et al., 2004).

In this study we used the ecotoxicogenomic approach to investigate the possible mode of action of some selected PPCPs, and to link these gene expression changes with physiological changes. Additionally we wanted to elucidate whether gene expression changes at low, environmentally realistic concentrations are a sensitive endpoint, and whether they can thus serve as early warnings of effects on the organism level.

1.6 Research goals

The aim of this thesis is to determine and assess effects and possible modes of action of selected human pharmaceuticals in *in vitro* systems with different fish cell lines (CYP3A, Chapter 2). Environmentally important PPCPs with interesting mode of actions, and of which only little is known about effects on aquatic organisms are focused on in this thesis. Two selected pharmaceuticals and the UV-filter EHMC were investigated *in vivo* in different developmental stages of zebrafish (Chapter 3 -5). The following research goals are thereby highlighted:

Optimisation. CYP3A alteration can serve as a biomarker of xenobiotic exposure. There are many microsome-based assays available to measure its enzyme activity *in vitro* and *in vivo*. However, these assays are quite time-consuming, as microsomes have to be initially prepared. Therefore, one goal of this thesis is to optimise an already available human whole cell assay for its use in fish. CYP3A enzyme activity was determined using the human CYP3A substrate 7-benzyloxy-4-trifluoromethylcoumarin (BFC) which is metabolized into a fluorescent product measurable by a plate reader (Chapter 2).

Investigation. External influences such as exposure to PPCPs can alter gene expression profiles in different developmental stages of organisms. These alterations can be measured using Agilent zebrafish whole genome oligonucleotide microarrays and qRT-PCR analysis. Therefore, we investigated the effects of diazepam (Chapter 3), PKC412 (Chapter 4) and EHMC (Chapter 5) on the gene expression to study their modes of action.

Validation. PPCPs mainly occur in the environment in the higher ng/L and lower µg/L range. As adverse effects at environmentally relevant concentrations are of concern, an additional goal of this thesis is to validate these effects on gene expression. Therefore, all studies were also conducted at low concentrations (Chapter 3-5).

Linkage and analysis. Alterations in gene expression do not always result in physiological effects. As there are many modification steps on the route from the gene to the protein, gene expression changes do not often result in protein alterations. Therefore, gene expression changes should be always linked to physiological and morphological parameters (Chapter 3, 4). To obtain these linkages, we performed additional experiments on several parameters including locomotor activity, gonad histology, angiogenesis, apoptosis, DNA damage and formation of reactive oxygen species.

Through this approach the hypothesis was tested that changes in gene expression may be more sensitive than behavioural, histological or other toxicological effects. Additionally, we addressed the question of whether gene expression changes propagate to toxico-physiological relevant measures such as development, behaviour (reduction of the ability to find food or to escape from predators), fertility and reproduction.

1.7 Outline of the thesis

The thesis is presented in 6 chapters, whereof chapter 2 to 5 contain the results obtained in different studies during this thesis.

Chapter 1: General introduction
In this chapter a general introduction to the topic and the thesis is given.

Chapter 2: A microtitre-plate based cytochrome P450 3A activity assay in fish cell lines
Alteration of CYP3A expression and activity in organisms can be an indication for exposure to xenobiotics. Therefore, these changes may serve as a biomarker of xenobiotic exposure. As no assays exist for measuring CYP3A activity in fish cells, we improved an existing human whole cell assay for its use in fish. This fluorescent CYP3A high-throughput assay uses 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as a substrate, which is then metabolized into a fluorescent product and can be measured. We show that this assay is suitable to analyse for induction as well as inhibition of CYP3A caused by pharmaceuticals.

Chapter 3: Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*

In this chapter we investigate the molecular effects of diazepam and identify its neurotoxic mode of action using an Agilent 4x44K zebrafish microarray. Additionally, alteration of gene expression of selected genes is analyzed in zebrafish larvae using qRT-PCR. In a second set of experiments behavioural studies are performed to analyze for any correlation between altered gene expressions and effects on the organism level. Furthermore, testis histology is conducted to link the down-regulation of *hsd17b3* with effects on spermatogenesis.

Chapter 4: Effects of the protein kinase inhibitor PKC412 on gene expression and link to physiological effects in zebrafish *Danio rerio* eleuthero-embryos

To identify molecular effects of the novel antineoplastic agent PKC412 (midostaurin), we applied Agilent 4x44K zebrafish microarray. Subsequently behaviour and physiological effects were investigated in order to analyze for correlations between altered gene-expression and effects on the organism level. Thereby, the locomotor activity, effects on vascular development, appearance of apoptotic cells, DNA damage and formation of reactive oxygen species were investigated.

Chapter 5: Global gene expression profile induced by the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) in zebrafish (*Danio rerio*)

The lipophilic UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) belongs to one of the most frequently used UV-filter in sunscreens, and accumulates in the aquatic food-chain. Despite its ubiquitous presence in water and biota, very little is known about its potential hormonal effects on aquatic organisms. Gene expression patterns of liver, testis and brain were investigated using Agilent 4x44K zebrafish microarrays. These changes were then analysed for linking gene expression to hormonal effects in order to investigate oestrogenic / antiestrogenic or androgenic / antiandrogenic activities.

Chapter 6: General discussion and conclusion

In the final chapter a general discussion is given, conclusions are drawn and an outlook is made.

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Chapter 2

A microtiter-plate-based cytochrome P450 3A activity assay in fish cell lines

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Abstract

Enzymes belonging to the cytochrome P450 3A (CYP3A) subfamily play an important role in the metabolism of endogenous substances and xenobiotics, including pharmaceuticals. Xenobiotics can alter CYP3A expression and activity, and therefore, changes in CYP3A activity may serve as a biomarker of xenobiotic exposure. To determine changes in CYP3A enzyme activity for environmental risk assessment of xenobiotics including pharmaceuticals, high-throughput assays are needed, but these are missing for fish cells to date. Here, we report on the development of a fluorescent-based CYP3A high-throughput assay for four fish cell lines cultivated in 96-well plates based on 7-benzyloxy-4-trifluoromethylcoumarin as a CYP3A substrate. We show that human CYP3A substrate BFC is catalyzed by fish CYP3A enzymes to a fluorescent product. Its formation is dependent on cell numbers and incubation time. Furthermore, we demonstrate that with this new CYP3A assay induction and inhibition of enzyme activity by pharmaceuticals can be determined. This new cell-based assay is suitable for detection of alteration in CYP3A enzyme activity in largescale experiments for screening of pharmaceuticals occurring in the environment.

Keywords: Cytochrome P450 3A, Fish cell lines, High-throughput assay, Pharmaceuticals

2.1 Introduction

Despite the occurrence of pharmaceuticals in the environment, little is known about their potential effects on ecosystems (Fent et al., 2006). Some pharmaceuticals have been investigated for their effects on aquatic organisms such as fish (Schwaiger et al., 2004; Runnalls et al., 2007), but it remains unclear whether dependable biomarkers of exposure and effects exist for human pharmaceuticals. Here, we propose the cytochrome P450 3A (CYP3A) enzyme as a potential candidate for exposure assessment in fish.

The CYP3A family is the largest subfamily of CYPs found in the liver and small intestine of mammals and fish (Hegelund and Celander, 2003; Lee and Buhler, 2003; Nallani et al., 2004). Cytochrome P450 3A plays an important role in the metabolism of endogenous substances and xenobiotics including pharmaceuticals. Induction of CYP3A expression by rifampicin and dexamethasone has been shown to occur in fish (Tseng et al., 2005). Cytochrome P450 3A was identified in several teleost species (e.g., CYP3A27 and CYP3A45 in rainbow trout (Lee et al., 1993; Lee and Buhler, 2003), CYP3A38 and CYP3A40 in medaka (Kullman et al., 2000; Kullman and Hinton, 2001), CYP3A30 and CYP3A56 in killifish (McArthur et al., 2003), and CYP3A65 in *Danio rerio* (Tseng et al., 2005)).

Because xenobiotics can influence CYP3A expression and activity, a change in CYP3A expression could serve as an indicator of exposure of an organism to them and provide information on their metabolism. There are several requirements for assays to be suitable as potential biomarkers. They should have a toxicological basis and short handling time, be low in costs, and, preferably, have a high-throughput potential. Alterations of CYP3A activity, respectively, mRNA level, are known as a suitable biomarker used for assessing contaminant exposure and effects.

Cytochrome P450 3A enzymes of mammals and fish exhibit similar catalytic properties due to structural similarities (Bork et al., 1989). In fish, they are regulated by the same receptors as in mammals, namely, through the pregnane X receptor (PXR) (Bresolin et al., 2005; Meucci and Arukwe, 2006; Milnes et al., 2008). The CYP3A expression can also be modulated by the constitutive androstane receptor, but the constitutive androstane receptor is restricted to mammals (Krasowski et al., 2005; Reschly and Krasowski, 2006). Due to these similarities, specific probes to determine CYP3A activity used in humans are supposed to be also specific for CYP3A activity in fish. Up to now, several substrates and their metabolites, respectively, have been used to determine CYP3A activity in mammals and in fish. The most widely used substrates to test CYP3A activity are testosterone (James et al., 2005) and erythromycin (erythromycin N-demethylase activity) (Vaccaro et al., 2005; Li et al.,

2008). Assays using these substrates have the disadvantage that they require time-intensive analytical tools such as high-performance liquid chromatography coupled with UV spectrophotometric detection or coupled with mass spectroscopy to investigate the metabolites. In addition, O-alkyl derivatives of resorufin, fluorescein, 7-hydroxycoumarins, and 6-hydroxyquinolines have been examined as substrates in mammals (Cohen et al., 2003). Commercial kits (P450- GloTM Assays, Promega) offer another possibility to measure CYP activity based on a luminescent method. These biochemical assays using purified components or subcellular fractions are designed to measure the activities of P450 enzymes from recombinant and native sources and can also be used for cellbased CYP450 induction assays. The assays contain different human substrates for different CYP450 enzymes such as CYP3A, CYP2C, etc.

Currently, only assays with testosterone hydroxylase, erythromycin N-demethylase, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), or aminopyrine N-demethylase as a substrate have been used to show CYP3A activity in microsomal preparations in fish (Stegeman et al., 1997; Machala et al., 1998; Thibaut et al., 2006). Thus far, there have been no whole-cell-based assays in microtiter plates for measuring CYP3A activity. Absorbance- or fluorescence-based assays in microtiter plates that do not require metabolite separation by chemical analytical techniques represent important progress and should be developed for large-scale monitoring of xenobiotics including pharmaceuticals.

Here, we present a CYP3A assay based on fluorescence measurement in a 96-well plate reader using fish cell lines suitable for large-scale measurements of CYP3A activity. Previous studies demonstrated that CYP1A can be induced by xenobiotics in *Poeciliopsis lucida* hepatoma cell-line 1 (PLHC-1) cells (Hahn and Chandran, 1996; Fent and Bätscher, 2000), zebrafish liver (ZFL) cells (Zeruth and Pollenz, 2005), and rainbow trout gonadal cell-line 2 (RTG-2) cells (Trattner et al., 2008). However, data on induction or inhibition of CYP3A enzyme activity and mRNA expression in fish are currently missing. The aim of the present study was to develop a versatile high-throughput assay for CYP3A activity in several fish cell lines, ZFL, RTG-2, and PLHC-1, using BFC as a substrate for potential use in studies of effects of pharmaceuticals, metabolism, and biomonitoring. We also analyzed whether pharmaceuticals can act as inducers or inhibitors of CYP3A-related enzyme activities in fish cell lines. Such information could facilitate the establishment of a new biomarker tool for pharmaceuticals, which can lead to induction or inhibition of CYP3A activity.

2.2 Materials and Methods

2.2.1 Chemicals

Dimethylsulfoxide (DMSO), acetonitrile (ACN), rifampicin, and BFC were purchased from Sigma-Aldrich. Phosphate buffered saline and diazepam were purchased from Roche Diagnostics.
Stock solutions of all pharmaceuticals were prepared in DMSO or ACN at a concentration of 100 µM if not otherwise stated. For the assay, stock solutions were diluted in cell culture medium resulting in a maximal solvent concentration of 0.1 %. Further concentrations were prepared by serial dilution.

2.2.2 Cells

ZFL. *Danio rerio* liver cells were obtained from the American Type Culture Collection (ATCC No. CRL-2643). The medium consisted of 50 % Leibovitz's L-15 medium (LuBioScience), 35 % Dulbecco's modified Eagle's medium with 4.5 g/L glucose (LuBioScience), and 15 % Ham's F12 (LuBioScience) (all without sodium bicarbonate) supplemented with 0.15 g/L sodium bicarbonate, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.01 mg/ml insulin, 50 ng/ml epidermal growth factor, and 5 % heat-inactivated fetal bovine serum (all from Sigma-Aldrich). The ZFL cells were grown at 28 °C in a humidified incubator. Cells were split usually every 7 d and subcultured at split ratios of approximately 1:3.

PLHC-1. *Poeciliopsis lucida* hepatoma cell-line 1 cells were grown in DMEM/F12 (LuBioScience) supplemented with 5 % fetal bovine serum (FBS; Sigma-Aldrich) in a humidified incubator with 5 % CO_2 at 30 °C. Cells were split usually every 4 d and subcultured at split ratios of approximately 1:6.

FHM. *Pimephales promelas* (fathead minnow) (FHM) cells were grown in DMEM/F12 (LuBioScience) supplemented with 5 % FBS (Sigma-Aldrich) and 20 mM HEPES at pH 7.2 (Sigma-Aldrich) at room temperature (21 ± 2 °C). Cells were split usually every 7 d and subcultured at split ratios of approximately 1:3.

RTG-2. *Oncorhynchus mykiss* (rainbow trout) gonadal cellline 2 (RTG-2) was obtained from the American Type Culture Collection (ATCC No. CCL-55). They were grown in DMEM/ F12 supplemented with 10 % FBS, 20 mM HEPES at pH 7.2 (Sigma-Aldrich) at room temperature (21 ± 2 °C). Cells were split usually every 7 d and subcultured at split ratios of approximately 1:3.

Huh7. The human hepatoma cells Huh7 were grown in DMEM/F12 with GlutaMAXTM (LuBioScience) supplemented with 5 % FBS in a humidified incubator with 5 % CO_2 at 37 °C. Cells were split usually every 4 d and subcultured at split ratios of approximately 1:6.

2.2.3 Determination of CYP3A65 catalytic activity

Assays performed in Eppendorf tubes. Approximately 2.5 million cells of each cell line were transferred to an Eppendorf tube and incubated in phenolred-free medium for 5 h. Phenolred- free medium was used to prevent the color of phenol red from interfering with spectrophotometric and fluorescence measurements in assays containing 50 µM BFC (Sigma- Aldrich) or ACN, respectively. 7-Benzyloxy-4-trifluoromethylcoumarin is metabolized by human CYP3A4 to give highly fluorescent 7-hydroxy-4-(trifluoromethyl)coumarin that can be detected readily spectrofluorometrically (Mensah-Osman et al., 2007). After 5 h, the cells were centrifuged for 4 min at 2,000 g at 4 °C. The supernatant was collected and transferred into a black 96-well plate (Huber). To stop the reaction, a stop solution (80 % ACN and 20 % 0.5 M Tris base) equal to 40% of the reaction volume was added. The plate was read on a GENios Tecan reader (Tecan) using an excitation wavelength of 410 nm and an emission wavelength of 530 nm. Each experiment was repeated three times.

Assays in 24-, 48-, and 96-well plates. Different numbers of cells were seeded in 24-, 48-, and 96-well plates and grown in culture medium. After 24 h, the medium was removed, and the cells were incubated in phenol-red-free medium containing 50 µM BFC or ACN, respectively, for 5 h. After this exposure time, the supernatant was transferred into a black 96-well plate and mixed with the stop solution, and the experiment was performed as described above.

Exposure to pharmaceuticals. Cells (~100,000 cells per well) were seeded in 96-well plates and grown in culture medium. After 24 h, the medium was removed, and cells were incubated in phenol-red-free medium containing the test pharmaceutical or the solvent control, respectively. The cells were incubated for an additional 24 h, and the CYP3A-assay was performed as described above. To control for equal numbers of cells in each well, the cells were removed from the well with trypsin and incubated with lysis buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton X- 100, and 0.1 % sodium dodecyl sulfate) to obtain whole cell extracts. From each whole cell extract, the protein content was determined with a Bradford assay (Bio-Rad Laboratories). Only wells with equal protein concentrations were analyzed for CYP3A activity.

Statistical analysis. Data were graphically illustrated with GraphPadH Prism 4 (GraphPad Software). Data distribution for normality was tested by Kolmogorov–Smirnov test, and the homogeneity of variance was tested by Levene's test. Differences between treatments were assessed by analysis of variance followed by a Bonferroni (Levene's test $p > 0.05$) test to compare the treatment means with the respective controls. In correlation tests, the different treatment groups were obtained by Pearson coefficients r. The results are given as mean ± standard deviation. Differences were considered significant at $p \leq 0.05$. All computations were performed with SPSS® 16.0 for Windows.

2.3 Results

2.3.1 Basic CYP3A activity in different fish cell lines

In the first set of experiments, we determined the CYP3A activity in vitro according to the protocol of Mensah-Osman et al. (Mensah-Osman et al., 2007). Our aim was to evaluate whether the human CYP3A substrate (BFC) is also a substrate for fish CYP3A enzymes. To control for known CYP3A activity, the human hepatoma cell line Huh7 was used. Figure 1 demonstrates that all evaluated fish cell lines showed basic CYP3A activity. The PLHC-1 and FHM cell lines displayed a more than 1.5-fold higher CYP3A activity than Huh7. The ZFL cells showed the lowest CYP3A activity, but the detected values were clearly above the background signals.

Treatment of cells with different drugs at different concentrations at the same time for monitoring the CYP3A activity is not feasible, so far. For this reason, we changed the existing protocol to a more efficient experimental system that allows the processing of multiple samples at the same time. For this procedure, approximately 100,000 cells per well were seeded in a 96-well plate. After cultivation of the cells for 24 h, they were incubated with the CYP3A substrate directly in the wells instead of trypsinizing the cells and transferring them into a 1.5-ml tube for incubation with the substrate. Hence, product formation was assessed with intact cells.

Figure 2A illustrates the two different procedures schematically (left panel: assay performed in 1.5-ml tubes; right panel: assay performed in microtiter plate). Figure 2B shows that CYP3A activity was detected when performing the assay in the wells. This is more elegant than performing the assay in a 1.5-ml tube. Because of fewer handling steps, more samples can be investigated in a shorter time, and there are fewer sources of potential handling mistakes.

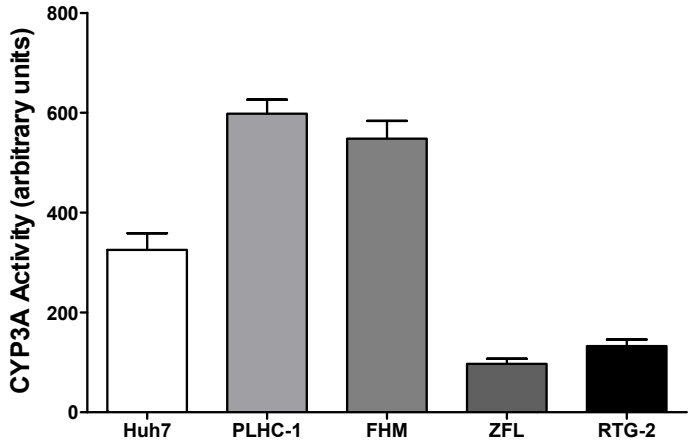

Figure 1

Endogenous cytochrome P450 3A (CYP3A) activity of the human hepatoma cells (Huh7), Poeciliopsis lucida cell-line 1 (PLHC-1), fathead minnow (FHM), zebrafish liver (ZFL), and rainbow trout (RTG-2) cells. Shown are results of at least three independent experiments ± standard deviation for each cell line. The CYP3A activity assays were performed in 1.5-ml tubes as described by Mensah-Osman et al.

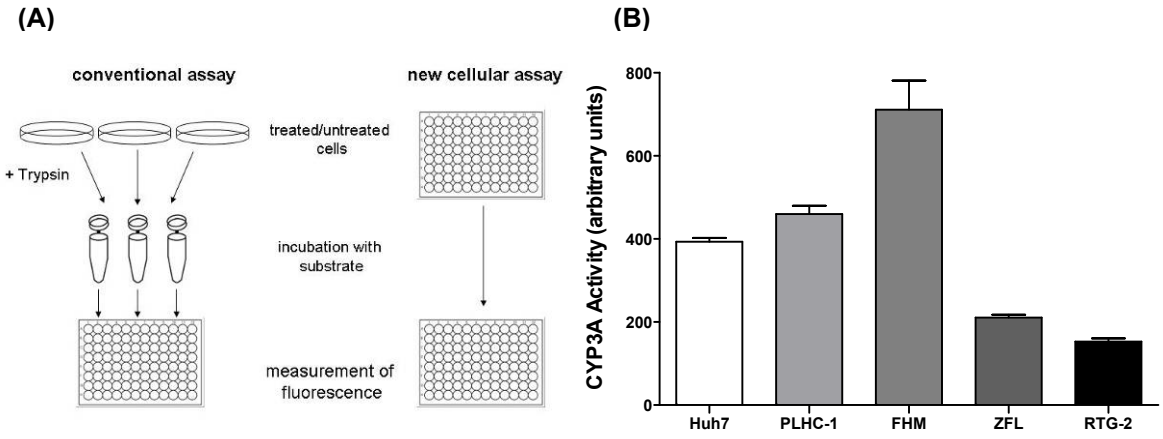

Figure 2

(A) *Diagram comparing the procedure of the cytochrome P450 3A (CYP3A) assay performed in 1.5-ml tubes (left) and our new cellular assay in 96-well plates (right).* ***(B)*** *Endogenous CYP3A activity obtained by performing the assay in 96-well plates. The CYP3A activity assay was performed in the human hepatoma (Huh7), Poeciliopsis lucida cell-line 1 (PLHC-1), fathead minnow (FHM), zebrafish liver (ZFL), and rainbow trout gonadal (RTG-2) cells. The incubation step was performed directly in the wells. Results of three independent experiments ± standard deviation for each cell line.*

Through the use of 96-well plates, the amounts of reagents used such as pharmaceuticals, cell media, and buffers can be reduced. Without trypsinization of the cells, the risk of inducing cellular stress responses that could alter the outcome of the assay is reduced. Hence, the results are more reproducible. The measured basic CYP3A activities in the different cell lines obtained by the new cell assay were comparable to the activities obtained

with the former protocol. Again, PLHC-1 and FHM cells showed the highest activity, and ZFL cells showed again much lower activity. From these experiments, we conclude that it is possible to perform the CYP3A activity assay directly in the culture dish without trypsinizing the cells.

2.3.2 Dependency of CYP3A activity on different conditions

To evaluate and validate the newly established CYP3A assay, different numbers of ZFL cells were seeded in 96- and 48-well plates. After 24 h of cultivation, the CYP3A activity assay was performed in the culture plates. Figure 3A demonstrates that increasing conversion of the CYP3A substrate into the fluorescent product was detected with increasing numbers of cells.

This clearly confirms that the detected fluorescence is based on CYP3A enzyme activity of the cells and not due to background fluorescence. Additionally, zebrafish cells grown overnight in a 96-well plate were incubated with CYP3A substrate, and the enzyme reaction was stopped at different time points. As shown in Figure 3B, there was a clear correlation between incubation time and amount of measured fluorescence. This result confirms again that the measured fluorescence signals originate from cellular enzyme activity.

2.3.3 Dependency of CYP3A activity on BFC concentration

As another control experiment to control the functionality of the CYP3A assay, we incubated ZFL and FHM cells with different concentrations of BFC (10, 20, 30, 40, and 50 µM). There was a clear correlation between measured fluorescence and concentration of BFC. The highest fluorescent signal was measured with 50 µM BFC (Figure 3C and D). This BFC concentration was used for all of the other experiments. As a proof of function of the enzyme in this new assay, the assay was stopped by protein precipitation with trichloroacetic acid. No conversion of the substrate was detectable when incubating cells with 10 % trichloroacetic acid for 1 h prior to the incubation with BFC. These results clearly show that only living cells and active enzyme, respectively, can cause substrate conversion (data not shown).

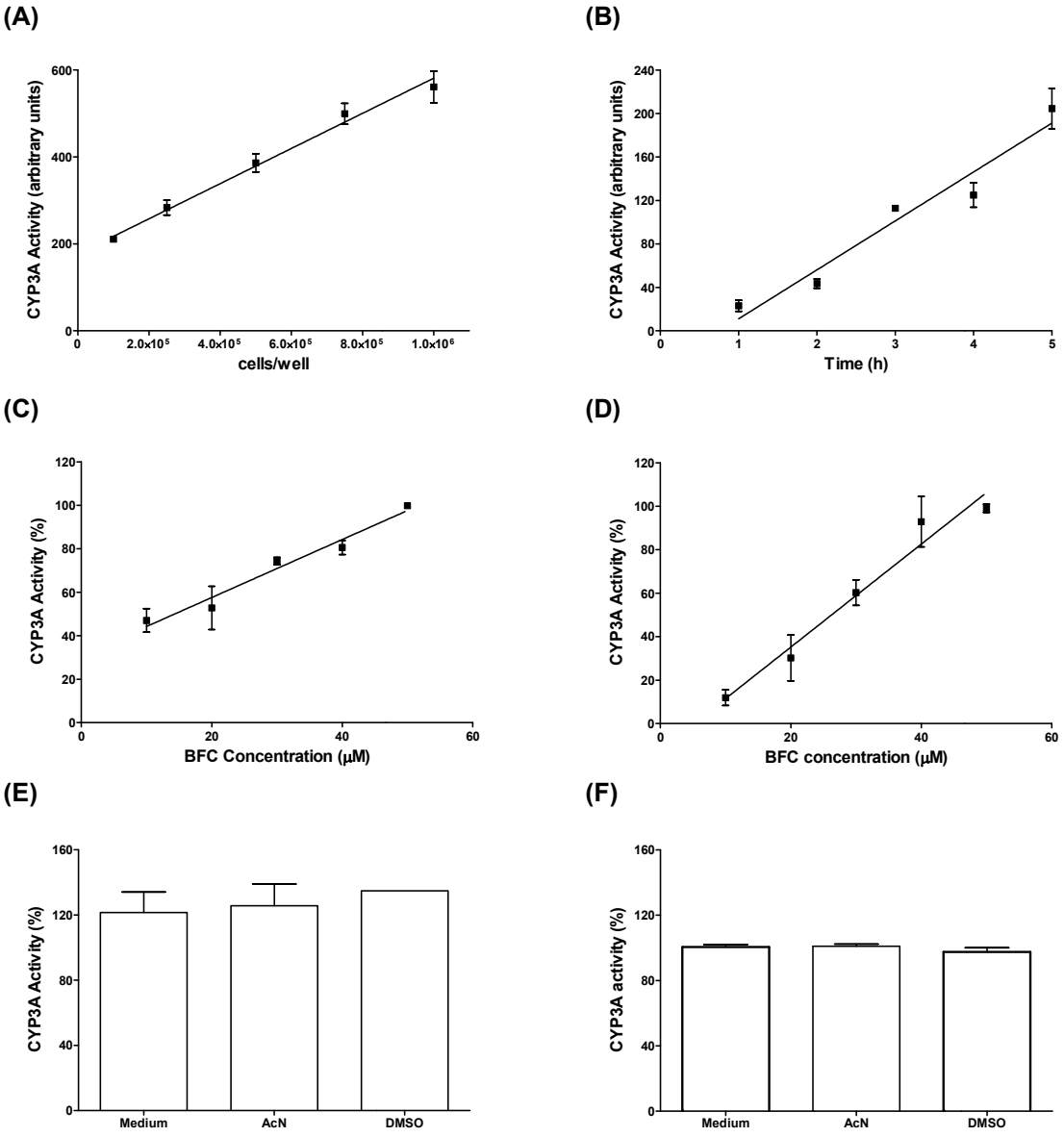

Figure 3

(A) *Correlation between measured fluorescence originating from cytochrome P4503A (CYP3A) enzyme activity and number of zebrafish liver (ZFL) cells (10^5–10^6, r^2 = 0.93). Results of three independent assays ± standard deviation.* ***(B)*** *Correlation between measured fluorescence and incubation time. The ZFL cells were incubated with CYP3A substrate 7-benzyloxy-4-trifluoromethylcoumarin (BFC), and the reactions were stopped after 1 to 5 h (r^2 = 0.88). Shown are the mean values of three experiments ± standard deviation (SD).* ***(C and D)*** *Correlation between measured fluorescence originating from CYP3A enzyme activity and different BFC concentrations. The ZFL (**C**, r^2 = 0.84) and fathead minnow (FHM) cells (**D**, r^2 = 0.84) cells were incubated with different concentrations of the CYP3A substrate BFC (10, 20, 30, 40, and 50 µM) for 5 h. Shown are mean values of three independent experiments ± SD.* ***(E and F)*** *No alteration of CYP3A activity through incubation with solvents. Human hepatoma (Huh7, **E**) and Poeciliopsis lucida cell-line 1 (PLHC-1, **F**) cells were incubated for 24 h with 0.1% dimethylsulfoxide (DMSO) and acetonitrile (ACN), respectively, to control for the influence of the solvents on CYP3A activity. No influence on CYP3A activity detectable. Shown are mean values of three independent experiments ± SD.*

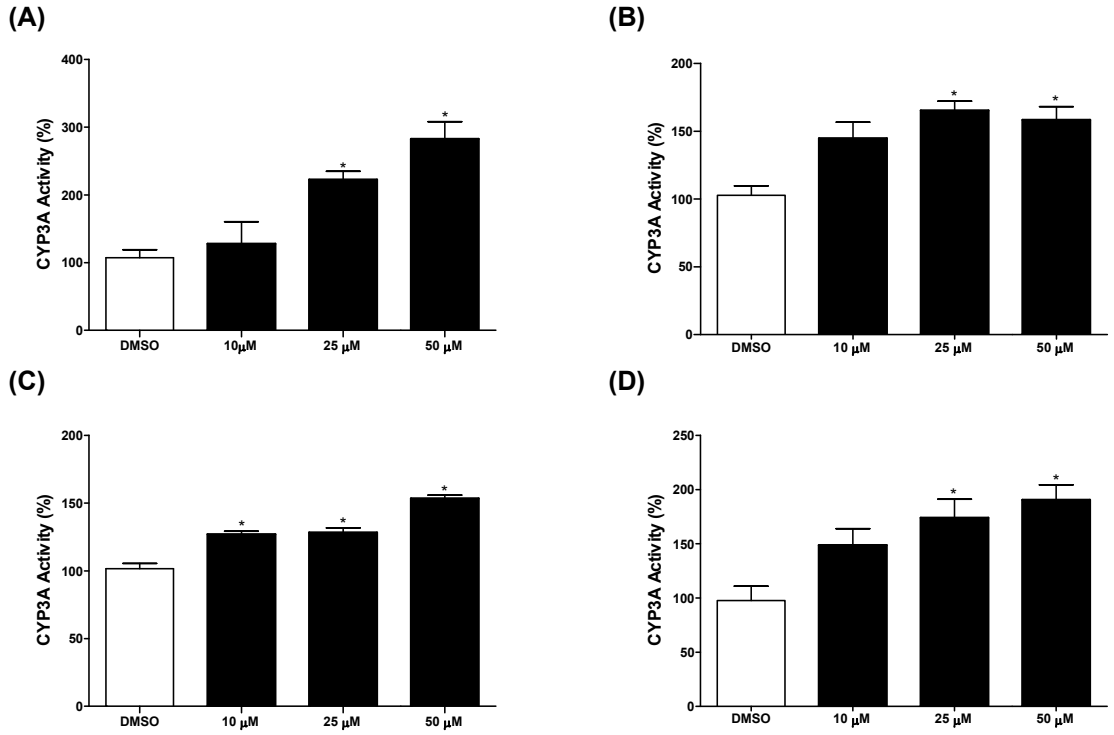

Figure 4

Induction of cytochrome P450 3A (CYP3A) activity by rifampicin in different cell lines: ***(A)*** *human hepatoma cells (Huh7),* ***(B)*** *Poeciliopsis lucida cell-line 1 (PLHC-1),* ***(C)*** *rainbow trout (RTG-2) cells,* ***(D)*** *fathead minnow (FHM) cells. Cells, treated with 10, 25, and 50 µM rifampicin for 24 h followed by CYP3A activity measurement. Shown are mean inductions of CYP3A activity of three experiments ± standard deviation. Differences in enzymatic activity between solvent controls with dimethylsulfoxide (DMSO) and rifampicin-treated cells were significant for 25 µM (p = 0.023) and for 50 µM (p = 0.001) in (**A**), for 25 µM (p = 0.004) and for 50 µM (p = 0.012) in (**B**), for 10 µM (p < 0.0001), for 25 µM (p < 0.0001), and for 50 µM (p < 0.0001) in (**C**), and for 25 µM (p = 0.042) and for 50 µM (p = 0.009) in (**D**).*

2.3.4 Induction and inhibition of CYP3A activity

To confirm the functionality of CYP3A enzymes in fish cells and to demonstrate the usefulness of the new CYP3A cellular assay, we evaluated the induction or inhibition potential of CYP3A enzyme activity by model compounds. First, we tested the influence of the solvents used on CYP3A activity. To this end, Huh7 cells and PLHC-1 cells, grown overnight in 96-well plates, were left untreated or treated for 24 h with 0.1 % ACN and 0.1 % DMSO prior to incubation. As shown in Figure 3E and F, the solvents had no effects on CYP3A activity. After confirming that solvents do not alter CYP3A activity, we treated the cells with the model compounds. For this purpose, PLHC-1, RTG-2, FHM, and control Huh7 cells, all grown separately in a 96-well plate, were treated for 24 h with 10, 25, and 50 µM rifampicin before performing the CYP3A activity assay in the plate. Treatment of Huh7 cells with rifampicin resulted in an increase in enzyme activity up to around 250 % compared with that of solvent control cells treated with DMSO (Figure 4A).

The treatment of PLHC1, RTG-2, and FHM with rifampicin also resulted in an induction of CYP3A enzyme activity (Figure 4B–D). In all three cell lines, the induction was up to 150 %. In addition to the induction of CYP3A activity, the inhibition of CYP3A activity was evaluated by treatment of PLHC-1 cells and Huh7 cells as controls with the known human CYP3A inhibitor diazepam. PLHC-1 and Huh7 cells grown in a 96-well plate were treated for 24 h with diazepam followed by CYP3A activity measurement. As shown in Figure 5, the CYP3Aactivity in PLHC-1 cells was inhibited up to 50 %; in Huh7 cells, the CYP3A activity was inhibited to 60 to 70 %.

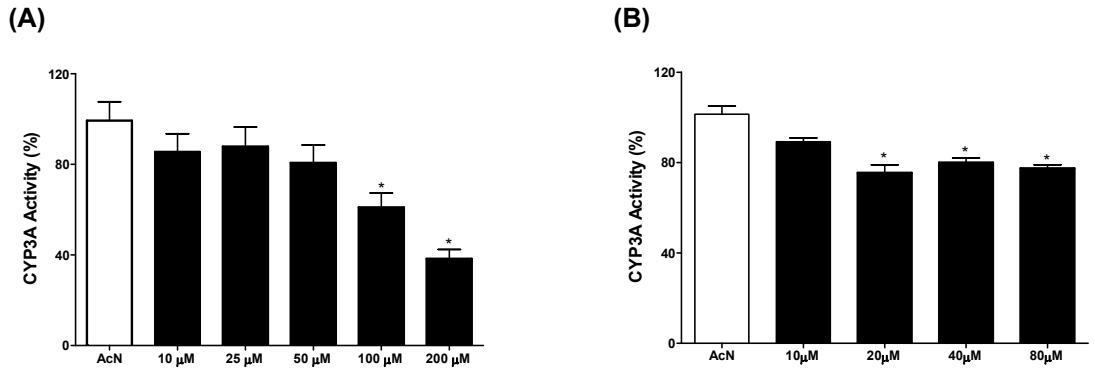

Figure 5

Inhibition of cytochrome P450 3A (CYP3A) activity by diazepam treatment of Poeciliopsis lucida cell-line 1 (PLHC-1) cells ***(A)*** *and human hepatoma (Huh7) cells* ***(B).*** *The CYP3A activity of PLHC-1 cells was inhibited significantly (p = 0.017) at 100 µM diazepam and at 200 µM (p < 0.0001) and at 20 µM (p = 0.002), 40 µM (p = 0.009), and at 80 µM (p = 0.004) in Huh7 cells.*

2.4 Discussion

The aim of our present study was to develop a highthroughput procedure that allows the analysis of the effects of many different environmental chemicals and pharmaceuticals on fish CYP3A activity at the same time. The basic principle of existing CYP3A activity assays is to incubate CYP3A enzymes with a specific substrate for a few hours. During this time, the substrate is converted by the CYP3A enzymes into a product that is determined afterward. Different CYP3A substrates exist for the incubation step, but all of them are substrates for human CYP3A. In the present study, we decided to work with the human CYP3A substrate BFC, which is catalyzed by CYP3A and small contribution of CYP1A2 (Stresser et al., 2000; Stresser et al., 2002), that is converted into a fluorescent product by CYP3A. We first wanted to know whether this human CYP3A substrate is also metabolized by CYP3A enzymes of different fish. For this reason, we adapted the protocol from Mensah-Osman et al. to evaluate whether the human-specific CYP3A substrate BFC (Renwick et al., 2000; Stresser et al., 2000) can also be catalyzed through fish CYP3A enzymes into the fluorescent product, as in

carp (Thibaut et al., 2006). As shown in Figure 1, fish CYP3A enzymes indeed can catalyze BFC. We demonstrate this in four cell lines from different tissues and different fish families. Surprisingly, the basal catalytic activities of PLHC-1 and FHM were even higher than that of the human Huh7, which was used as a control. The ZFL and RTG-2 cells showed lower enzyme activities than the human cells, but the detected signals were above the background level. After demonstrating that the human substrate is also a substrate for fish cells, we modified the existing protocol so that the whole procedure can be completely performed on microtiter plates. The cells were seeded on a 96-well plate, and after recovery overnight, the substrate BFC was added directly to the well. After incubation, the supernatant of each well was transferred to a new 96-well plate, loaded already with Stopp buffer, and the plate was read on a spectrofluorophotometer. Figure 2 demonstrates the same pattern of CYP3A activity as with the original protocol. Again, PLHC-1 and FHM cells showed higher CYP3A activity than Huh7 cells, and ZFL and RTG-2 cells again showed the lowest CYP3A activity. To verify that we actually measured CYP3A activity, we performed a number of control experiments to evaluate the effects of different cell numbers, different incubation times with the substrate, and different concentrations of the substrate. Figure 3A shows a clear correlation between the number of cells and measured fluorescence. There is also a good correlation between measured fluorescence and incubation time (Figure 3B): The longer the incubation time was, the higher the amount of fluorescence. The best results were obtained when the incubation time with the substrate was 5 h. This confirms that an enzyme activity was measured and not an artificial signal. We could also demonstrate a correlation between the concentration of BFC used and the measured fluorescence after incubation (Figure 3C and D). The highest fluorescence signal was detected when the cells were incubated with 50 µM BFC. These results confirm again that we measured enzyme activity and not an artificial signal. Because we want to use this assay to evaluate the effects of different environmental pollutants and pharmaceuticals on CYP3A activity, we performed control experiments with the solvents that will be used later to dissolve the compounds. For this purpose, cells were incubated with 0.1 % solvent (DMSO or ACN) for 24 h, and the CYP3A activity was measured afterward. As shown in Figure 3E and F, there is no influence of the solvents on CYP3A activity. With all of these control experiments, we were able to set up the optimal conditions, namely, 5 h of incubation with 50 µM BFC, for the CYP3A assay that were used later for induction and inhibition experiments. To strengthen our newly developed assay, the effect of inducers and inhibitors on CYP3A activity was assessed. The CYP3A inducer rifampicin led to induction of CYP3A activity in PLHC-1 (Figure 4B), RTG-2 (Figure 4C), and FHM (Figure 4D) cells, being significant already at 10 µM rifampicin. Compared with human control cells that show induction of enzyme activity up to 350 %, the fish CYP3A enzymes which show an induction up to 150 % are obviously less inducible than the human

CYP3A. In addition to the induction of CYP3A activity, our new cell assay is also suitable to detect an inhibition of enzyme activity by pharmaceuticals. A clear dose-dependent inhibition of the enzymatic activity is found with diazepam (Figure 5). Therefore, our cell assay is not only suitable for determining induction of CYP3A activity but also for detecting its inhibition. Our results clearly show that our modified protocol can be used to detect alteration in CYP3A activity by chemicals. The overall advantage of our assay lies in the fact that the whole procedure can be performed on microtiter plates. Specifically, different compounds or different concentrations of one compound can be investigated on one plate at the same time; most of the steps can be performed using multichannel pipettes, which reduces handling time and minimizes pipetting mistakes and moreover has the potential for adaption for a pipetting robot; and the assay is not expensive compared with existing CYP3A kits on the market. This assay can be used as a first screening step in the environmental risk assessment of pharmaceuticals. With this method, pharmaceuticals or chemicals can be screened easily with regard to their abilities to alter CYP3A activity. Identified compounds can be examined further, including the quantification of CYP3A activity. Another interesting potential of this assay is the comparison of different sensitivities of different fish species to environmental contaminants in regard to CYP3A activity alterations. The CYP3A activity and expression are regulated by PXR (Bresolin et al., 2005; Meucci and Arukwe, 2006). The PXR is a member of the nuclear hormone receptor superfamily. Pregnane X receptor functions as a ligand-activated transcription factor and regulates the metabolism, transport, and excretion of exogenous compounds, steroid hormones, vitamins, bile salts, and xenobiotics. Pregnane X receptor genes have been cloned and functionally characterized from a variety of vertebrate species, including human, rhesus monkey, mouse, rat, rabbit, dog, pig, chicken, frog, and zebrafish (Reschly and Krasowski, 2006). Like other nuclear receptors, PXRs have a modular structure with two major domains: an N-terminal DNA-binding domain and a larger C-terminal ligand-binding domain (LBD). The PXR LBD is unusually divergent across species, compared with other nuclear receptors, with only 50% sequence identity between mammalian and nonmammalian PXR sequences; other nuclear receptors tend to have corresponding sequence identities at least 10 to 20 % higher. Even the PXR DNA-binding domain, which is more highly conserved across species than the LBD, shows more cross-species sequence diversity than other nuclear receptors (Krasowski et al., 2005; Reschly and Krasowski, 2006). Due to this diversity, we would expect different sensitivities of different fish species to pharmaceuticals in regard to the alteration of CYP3A activity. Future steps will be the use of this novel assay to screen pharmaceuticals for their abilities to alter CYP3A activity in different fish cell lines. This screening will help to identify compounds of ecotoxicological interest that subsequently will be investigated further.

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V. Christen and D. Oggier contributed equally to this work.

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Chapter 3

Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*

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Abstract

We applied zebrafish whole genome microarrays to identify molecular effects of diazepam, a neuropharmaceutical encountered in wastewater-contaminated environments, and to elucidate its neurotoxic mode of action. Behavioral studies were performed to analyze for correlations between altered gene expression with effects on the organism level. Male zebrafish and zebrafish eleuthero-embryos were exposed for 14 d or up to 3 d after hatching, respectively, to nominal levels of 273 ng/L and 273 µg/L (determined water concentrations in the adult experiment 235 ng/L and 291 µg/L). Among the 51 and 103 altered transcripts at both concentrations, respectively, the expression of genes involved in the circadian rhythm in adult zebrafish and eleuthero-embryos were of particular significance, as revealed both by microarrays and quantitative PCR. The swimming behavior of eleuthero-embryos was significantly altered at 273 µg/L. The study leads to the conclusion that diazepam-induced alterations of genes involved in circadian rhythm are paralleled by effects in neurobehavior at high, but not at low diazepam concentrations that may occur in polluted environments.

Brief: Effects of diazepam on gene expression and behavior in different life stages in zebrafish

3.1 Introduction

In the last decade, steadily increasing levels of aquatic environmental residues of human and veterinary pharmaceuticals have continuously alerted the scientific community. As a result, multiple research efforts have been initiated to scrutinize possible input pathways and fate of these newly emerging pollutants. Additionally, effects on non-target species have been studied to protect prospectively the environmental health and to minimize exposure risks (Fent et al., 2006; Ankley et al., 2007; Kümmerer, 2008). Pharmaceuticals active on the brain and nervous system functions, including antidepressants, antipsychotics and anaesthetics are of direct environmental relevance because of their potential effects on non-target species. Based on evolutionarily conserved pharmacological targets, receptors and cellular biomolecules, environmental pharmaceuticals have been shown to provoke adverse effects such as hormonal dysfunction and neurotransmission disruption (Jones et al., 2007; Gunnarsson et al., 2008; Kostich and Lazorchak, 2008).

A prominent representative pharmaceutical of this group is the environmental contaminant and highly prescribed neuropharmaceutical diazepam. Diazepam is the active ingredient of Valium® and belongs to the benzodiazepine group, which has widespread application in human medicine (Jouvel et al., 2000). Concentrations of up to 0.66 µg/L (van der Hoeven, 2004) have been measured in effluents of sewage treatment plants. In surface water, concentrations have been found ranging from 0.04 µg/L in Germany (Ternes, 1998) to 0.66 µg/L in Belgium (van der Ven et al., 2004). This substantial environmental exposure may lead to negative effects on non-target species.

Benzodiazepines mechanism is through binding to the GABA receptor resulting in central nervous system (CNS) depression. In addition, diazepam has also anxiolytic, sedative, muscle-relaxant, anti-convulsive and anti-epileptic properties (Shader and Greenblatt, 1977). As in mammals, three subtypes of GABA target receptors ($GABA_A$, $GABA_B$ and $GABA_C$) occur in fish, and the binding site for benzodiazepines in the fish brain is suggested to be highly conserved between fish and mammals (Carr and Chambers, 2001). In non-target species acute diazepam toxicity in the high and chronic toxicity in the low mg/L range (Straub, 2008). The measured "no observed effect concentration" (NOEC) of diazepam in early life stage tests with *D. rerio* was found to be 273 µg/L, and the "lowest observed effect concentration" (LOEC) was found to be 2.57 mg/L (Straub, 2008). Exposure of fish to diazepam may impair locomotion (Zhdanova et al., 2001; Nunes et al., 2008), affect vision (Salas et al., 1992) and can produce anxiolytic effects at concentrations between 1.25 to 5 mg/L, doses that do not cause sedation (Bencan et al., 2009). Nevertheless, there is a lack of chronic environmental toxicity studies concentrating on the modes of action on this

residual pharmaceutical (Fent et al., 2006). Furthermore, proper ecological risk assessments of diazepam at environmentally realistic concentrations in aquatic organisms have to be further investigated.

Classical ecotoxicological tests, e.g. OECD guideline 204 (prolonged 14 d fish test), are important for risk assessment, but are often not sensitive enough to identify the subtle effects of pharmaceuticals. Adverse effects of pharmaceuticals may be more accurately determined by focusing on mode of action (Fent et al., 2006; Owen et al., 2007; Runnalls et al., 2007). Based on these considerations, the environmental consequences of diazepam deserve further attention, in particular as effects of diazepam on the global gene expression profile in aquatic organisms are unknown.

In our present study, we determine the neurotoxic mode of action of diazepam in zebrafish to clarify potential molecular effects of this drug by analyzing the global gene expression after exposure for 14 d exposure. This toxicogenomics approach allows the identification of several thousand genes and the corresponding expression profiles upon drug exposure which will assist in the elucidation of both the molecular effects and the compound's modes of action (Robbens et al., 2007).

The aim of our study was to focus on the modes of action in the brain and in eleuthero-embryos at low diazepam concentrations using a whole genome oligonucleotide microarray for zebrafish. By analyzing diazepam at the NOEC of zebrafish eleuthero-embryos (273 µg/L) we compared the effects on the transcriptional level with known effects in the early life stages test (Straub, 2008). Our hypothesis is that the response of zebrafish to diazepam exposure is similar to the human response, as the GABA receptors are evolutionary highly conserved. On this basis, we searched for alteration of gene expression patterns associated with anxiolytic or sedative effects. Thereby, we hypothesize that alterations in gene expression - because of multiple endpoints and mechanistic information - are more sensitive than physiological or morphological parameters (although they are perhaps less ecologically relevant). Moreover, we compared the global gene expression pattern in the brain to the behavior of zebrafish to analyze, whether the observed molecular effects may correlate with and propagate to higher levels of biological organization. In addition, gene expression analysis may also reveal unknown regulatory mechanisms in fish not directly related to the modes of action of diazepam in humans.

3.2 Materials and Methods

3.2.1 Chemicals

Diazepam (≥ 99%, MW: 284.74, logK_{OW}: 2.82-2.99) was kindly supplied by F.Hoffmann- La Roche Ltd (Basel Switzerland). Acetonitrile was purchased from Brunschwig (Basel,Switzerland), methanol and isopropanol were from Stehelin (Basel, Switzerland). NaH_2PO_4, Na_2HPO_4, HCl, chloroform, ammonium hydroxide and phosphoric acid were obtained from Sigma Aldrich (Buchs, Switzerland). Paraffin tissuewax, xylol, UltraClear and hematoxylin were purchased from Medite (Nunningen, Switzerland) and eosin from Carl Zeiss AG (Feldbach, Switzerland).

3.2.2 Maintenance of zebrafish

Juvenile zebrafish (*Danio rerio*) were obtained from Harlan Laboratories (Itingen, Switzerland). They were transferred to culture tanks (300 L) and raised for 4 months in our laboratory. Fish were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 µS/cm. The water temperature was held constant at 27±1 °C with the photoperiod set at 16:8 h light/dark. Fish were fed twice daily with TetraMin pellets (Tetra GmbH, Melle, Germany) *ad libitum*, once a day with brine shrimp (*Artemia salina*) and twice a week with *Daphnia magna*.

3.2.3 Exposure experiment

Exposure of adult males. The experimental setup consisted of four replicates of water controls and two diazepam doses. Twenty male zebrafish per replicate were held in 20-L stainless steel tanks in well-aerated exposure water. Fish were exposed for 14 d to the nominal concentrations of 273 ng/L and 273 µg/L diazepam, respectively. The lower concentration (273 ng/L) was assumed to represent the worst case environmental concentration, and 273 µg/L refers to the NOEC in the early life stages test (Straub, 2008). During the experiment fish survival, appearance and behavior were determined.

A 48 h static-renewal procedure was used to minimize handling stress for the fish. Thereby after 24 h, food remains and feces were removed by siphoning 1/3 of the water and replacement by new exposure water containing the appropriate diazepam concentrations. After 48 h, the water renewal procedure was repeated, but this time by replacing total tank water (20 L). The quality of the exposure water was continuously monitored by the oxygen concentration determination (>70%), the pH value (6.7-7.2) and the temperature (27±1 °C).

Mortality and abnormal behavior were recorded daily. At the end of the experiment (day 14), all fish were anaesthetized in a clove oil solution (Fluka AG, Buchs, Switzerland). Individual fish length and weight was measured in order to assess the condition factor (CF=weight(g)/length(mm) x 100). The brain of each fish was dissected after 14 d and stored in RNA*later*. A total of 15 brains were pooled for microarray analysis and for quantitative real-time polymerase chain reaction (qRT-PCR) confirmation.

Eleuthero-embryo exposure. The static exposure setup consisted of six replicates of water control and two diazepam doses. A total of 15 fertilized eggs per replicate were exposed up to 3 d after hatching to nominal concentrations of 273 ng/L and 273 µg/L diazepam, respectively. Every 24 h, lethal and sublethal effects were evaluated and dead eleuthero-embryos were removed. The quality of the exposure water was continuously monitored by oxygen concentration determination (>70%), the pH value (6.7-7.2) and the temperature (27±1 °C). At the end of exposure, eleuthero-embryos were anaesthetized a clove oil solution. A total of 15 eleuthero-embryos per replicate were pooled in RNAlater for qRT-PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland).

3.2.4 Chemical analysis

To determine actual exposure concentrations, 250-mL aliquots of exposure water were taken during the experiment for diazepam concentration analysis. Water samples of each treatment group were taken at the beginning (0 h), before siphoning (24 h) and prior to full water renewal (48 h). This was done on days 1 to 3, 7 to 9 and 11 to 13, respectively, from different randomly selected replicate tanks. The water samples were stored at -20 °C until analysis by HPLC. Extraction of water samples was performed according to (Cordero and Paterson, 2007) and chemical analysis according to (Rouini et al., 2008). The chemical analysis was performed separately for the microarray experiment and the behavior experiment. Due to limited amounts of exposure water, the concentration in the exposure water of the eleuthero-embryos experiment could not be measured.

3.2.5 RNA isolation, array hybridization and sample selection

Total RNA was extracted from zebrafish brain pools using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 UV-VIS Spectrophotometer at 260 nm. The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing a 260/280 nm ratio between 1.8-2.1, a 28S/18S ratio between 1.5-2 and an RNA integrity number (RIN) > 8 were processed further. A total of 12

arrays (Agilent 4 × 44 K Zebrafish microarray) were used, including four for the control group, four for the 273 ng/L and four for the 273 µg/L diazepam dose group. Total RNA samples (600 ng) were reverse-transcribed into double-strand cDNA in the presence of RNA poly-A controls with the Agilent One-Color RNA Spike-In Kit.

3.2.6 Cy3 labeling and hybridization

After reverse-transcription of RNA into double-stranded cDNA, double-strand cDNA was *in vitro* transcribed into cRNA in the presence of Cy3 labeled nucleotides using a Low RNA Input Linear Amp Kit +Cy dye (Agilent Technologies, Basel, Switzerland), performed at the Functional Genomic Centre of ETH and University of Zürich, Switzerland. The Cy3-labeled cRNA was purified using an RNeasy mini kit (Qiagen, Basel, Switzerland), and quality and quantity was determined using a NanoDrop ND-1000 UV-VIS Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Only cRNA samples with a total cRNA yield higher than 2 µg and a dye incorporation rate between 9 pmol/µg and 20 pmol/µg were used for hybridization. Cy-3-labeled cRNA samples (1.65 µg) were mixed with Agilent blocking solution, subsequently fragmented randomly to 100-200 bp at 65 °C with fragmentation buffer and resuspended in hybridization buffer as provided by the gene expression hybridization Kit (Agilent Technologies). Target cRNA samples (100 µL) were hybridized to the Agilent Zebrafish 4x44K Gene Expression Microarray for 17 h at 65 °C. The hybridized arrays were then washed using Agilent GE wash buffers 1 and 2 according to the manufacturer's instructions and scanned by an Agilent Microarray Scanner (Agilent p/n G2565BA) at 5 µm resolution with the green photomultiplier tube set to 100% and a scan area of 61 x 21.6 mm. Image generation and feature extraction was performed using the Agilent Feature Extraction (FE) software version 9.5.3. Quality control was additionally considered before performing the statistical analysis. These included array hybridization pattern inspection: absence of scratches, bubbles, areas of non-hybridization, proper grid alignment, spike performance in controls with a linear dynamic range of 5 orders of magnitude and the number of green-feature non-uniformity outliers which should be below 100 for all samples.

3.2.7 qRT-PCR analysis

Eight differentially expressed genes of interest were selected for microarray result confirmation using qRT-PCR. Gene-specific primers were designed based on published zebrafish sequences (Table 1). Total RNA from a pool of 15 zebrafish brains and a pool of 15 eleuthero-embryos was isolated as described above (n = 5 replicates for adults, and n = 6 replicates for eleuthero-embryos).

1 µg of total RNA template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min.

Table 1

Primer sequences for quantitative real-time PCR analysis.

Probe ID	Gene	Sequence Accession #	dir	Sequence	Ampl. size
-	*RpL13α*	NM_212784	fw rv	agc tca aga tgg caa cac ag aag ttc ttc tcg tcc tcc	100 bp
A_15_P121171	*arntl2*	NM_131578.1	fw rv	gtg tca acc aac acg gtt gta tcc tgg aac ttg ttg gga ttt ctt ggc	120 bp
A_15_P106879	*arr3*	NM_200792	fw rv	cat gat ggg agg tgg act ct act tgc tcc tca ctg gct gt	140 bp
A_15_P173191	*cry2b*	NM_131792	fw rv	aaa cga cgc atc gtc tta tag aat tcg tgg ttg tgg tcc g	138 bp
A_15_P103946	*cry5*	NM_131788.1	fw rv	cat gga gag aac gaa ctg gg gtg cag aca agc agc cga ac	115 bp
A_15_P191501	*gabrr*	OTTDART00000004249	fw rv	acg tca tgc gag tgc tcg tcg tca gta tag gcg tag	151 bp
A_15_P104177	*hsd17b3*[a]	NM_200364.1	fw rv	ttc acg gct gag gag ttt g gga ccc agg tag gaa tgg	121 bp
A_15_P187316	*nr1d1*	NM_205729.1	fw rv	gtg aac aac cag ctg cag aa act gta agg cct gga cat gg	125 bp
A_15_P236266	*per1*	NM_001030183.1	fw rv	atg cgt gca aga agt ggt g acg tcc tca ttt agc gga ctc	131 bp

arntl2 (aryl hydrocarbon receptor nuclear translocator-like 2), ***arr3*** (arrestin 3, retinal), ***cry2b*** (Cryptochrome 2b), ***cry5*** (Cryptochrome 5), ***gabrr*** (gamma-aminobutyric acid (GABA) receptor, rho 1), ***hsd17b3*** (hydroxystereoid (17-beta) dehydrogenase 3), ***nr1d1*** (nuclear receptor subfamily 1, group d, member 1), ***per1*** (period homolog 1), **a** Hoffmann et al., 2008

The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). The following PCR reaction profile was used: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec, and 61 °C for 60 sec followed by a melting curve analysis post run.

The delta CT value was derived by subtracting the threshold cycle (CT) value for the housekeeping gene (*RpL13α*), which served as an internal control, from the CT value of the target gene, respectively. All reactions were run in duplicate using the Biorad CFX96

RealTime PCR Detection System (Biorad, Reinach, Switzerland). The mRNA expression level of the different genes was expressed as fold-increase according to the formula:

$2^{\Delta CT(\text{untreated sample})- \Delta CT(\text{treated sample})}$.

PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled brain cDNA (r^2>0.96 for all primers). Due to the high efficiency no corrections in the calculation of the delta CT were necessary.

3.2.8 Measurement of locomotor activity

Multispecies Freshwater Biomonitor®. Diazepam effects on locomotor activity were measured in both male zebrafish and zebrafish eleuthero-embryos. Fish activity was observed using flow-through test chamber with quadrupole impedance conversion as measuring device, connected to a measuring unit and personal computer with data analysis software (Gerhardt et al., 1994).

Measurement chambers, made of an acryl glass cylinder sealable on both ends, with a size of 15 cm in length and a diameter of 5 cm for the adult fish, and of 4 cm in length and a diameter of 2 cm for the eleuthero-embryos, allowed free movement during measurement. To measure locomotor activity, 12 male zebrafish were exposed to nominal concentrations of 273 ng/L (measured 330 ng/L) or 273 µg/L (measured 260 µg/L) diazepam in addition to a water control for 14 d in stainless steel aquaria as described above. After 3 d and 14 d of diazepam exposure the animals were placed into the measurement chambers placed in a stainless steel aquaria filled with 15 L uncontaminated water. The chambers themselves were laid horizontally on the bottom of the test tanks. Following an acclimation time of 2 h measurements were started and behavior of 12 male zebrafish per treatment was continuously recorded at intervals of 10 min for a 24 h duration. Behavioral recording during these intervals lasted for 4 min each.

For eleuthero-embryos locomotor activity measurements, fertilized eggs were exposed to 273 ng/L or 273 µg/L diazepam as described above. Locomotor activities of hatched eleuthero-embryos were assessed 3 d after hatching for 20 eleuthero-embryos per diazepam dose and the water control, respectively, as described above. Following an acclimation phase of 10 min measurements were started and behavior was monitored with a recording duration lasting 4 min for a period of 2 h with intervals of 10 min each. For locomotor activity

measurements, means of locomotor activities (% time spent on locomotion) for each fish were calculated for two hours during the 2-h or 24-h time period.

3.2.9 Histological analysis of testes

The testes of 14-19 male fish per group were examined histologically to reveal diazepam induced changes. Whole animals were fixed in neutral buffered formalin (Roth, Arlesheim, Switzerland), dehydrated in graded ethanol and xylene, paraffinized in a tissue processor (Gewebeentwässerungsautomat TPC 15 Duo; Medite, Nunningen (Switzerland)) and embedded in paraffin wax. Cross sections of 4.5 µm thickness were stained with hematoxylin and eosin. Sections were examined microscopically to determine the fish reproductive condition (staging according to Schulz et al 2009). The testes were analyzed histomorphometrically (Olympus BX41, Olympus Schweiz AG, Volketswil, Switzerland) by counting and categorizing the spermatogenic cysts (spermatogonia, spermatocytes, mature spermatozoa) at 40x magnification.

3.2.10 Data analysis and statistics

Raw microarray data was analyzed using the GeneSpring GX 10 software (Agilent Technologies). In a first step, the Agilent Feature Extraction software output was filtered on the basis of feature saturation, non-uniformity, pixel population consistency and signal strength relative to back ground level (Agilent Feature Extraction Manual). Only positively marked entities, in which at least 50% of the values for any out of the three conditions were accepted for further evaluation. All data was quantile normalized. In a second step, several quality control steps (e.g. correlation plots and correlation coefficients, quality metric plots and PCA) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality.

Differentially expressed genes from the microarray were determined using a Benjamini-Hochberg multiple correction-ANOVA test ($p<0.05$), followed by a TukeyHSD post-hoc test. The genes were considered differentially expressed when $p<0.05$ and the fold change (FC)≥2. To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneSpring GX 10 was used. Only those categories where $p<0.05$ were considered differentially altered. MetaCore TM (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the differentially expressed genes in specific pathways (FDR< 0.05). The microarray data used in this analysis have been submitted to NCBI GEO database under accession number GSE23157 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nfudjckukeakytk&acc=GSE23157).

Data from qRT-PCR, locomotor activity and histology were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov-Smirnov test and the variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p<0.05$) to compare treatment means with respective controls. Results are given as mean ± standard error of mean. Differences were considered significant at $p \leq 0.05$. #

3.3 Results and Discussion

3.3.1 Alteration of gene expression by diazepam

Diazepam concentrations in the exposure water were measured at 0, 24 and 48 h in order to determine the actual exposure concentrations (Table 2). At nominal 273 ng/L, diazepam concentration was 243±16 ng/L (n=3) at 0 h and decreased to 225±30 ng/L (n=3) at 48 h, but this was not significant. At 273 µg/L, diazepam decreased from 315±16 µg/L (n=3) to 272±6 µg/L (n=3). The geometric mean of actual exposure concentrations were 235 ng/L and 291 µg/L. In none of the treatment groups, mortalities occurred. No significant differences on condition factors (CF) were noted (Table 3, Figure 1).

Table 2

Nominal concentration and measured concentrations of diazepam in exposure waters after 14 days of exposure

Nominal concentration	**Exposure waters**						
	Measured concentration						
	0h	**% of nominal**	**24h**	**% of nominal**	**48h**	**% of nominal**	**Geometric Mean**
273 ng/L	242.9±16 ng/L	89	236.8±23 ng/L	87	225.4±30 ng/L	82	235 ng/L
273 µg/L	314.9±16 µg/L	115	287.6± 10 µg/L	101	272.4±6 µg/L	104	291 µg/L

Table 3

Body weight, length and Condition Factor (CF) of exposed fish after 14 days of exposure (n = 20 males per treatment) to 235 ng/L and 291 µg/L diazepam.

	Exposure (µg/L)	Survival (%)	Male		
			Body weight (mg)	Body length (mm)	CF
Control	Water	100	372.97 ± 15.1	36.54 ± 0.4	0.76 ± 0.01
Diazepam	0.235	100	364.48 ± 5.2	35.92 ± 0.3	0.79 ± 0.02
	291	100	372.35 ± 23.5	35.73 ± 0.7	0.82 ± 0.01

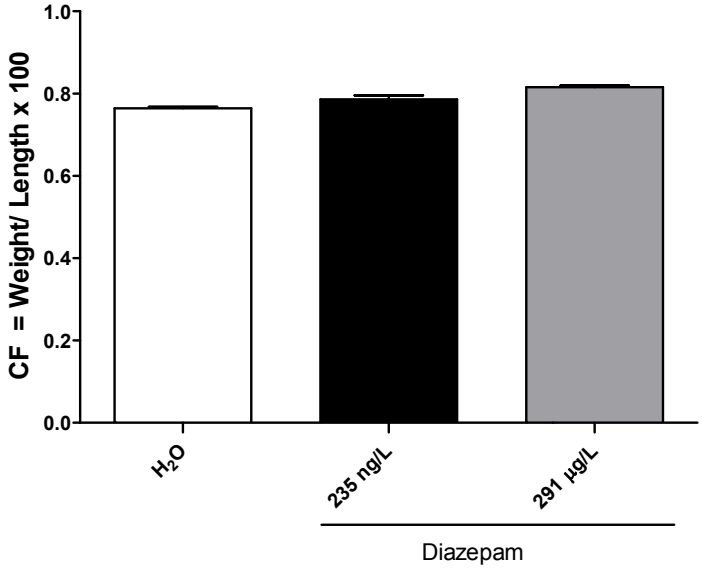

Figure 1

Condition factor (CF) of male zebrafish. Controls (white bar) and fish exposed to 235 ng/L and 291 µg/L diazepam (black and grey bar). Values are means ± SEM (n = 20 males). Measured median diazepam concentrations are given.

Gene expression profiles derived from microarray results of control and diazepam-exposed adult zebrafish brains (control, n = 3; diazepam, n = 4) are based on 15 pooled individuals. To test the data quality normalized data were then subjected to a PCA. As shown in Figure 2, the replicates of the 235 ng/L diazepam treatment group differ less from the control than the replicates from the 292 µg/L diazepam treatment group. The similarity to the control group is also reflected in the fact that fewer genes were significantly regulated in the lower concentration (Appendix 1 Table 1).

As listed in Appendix 1 Table 1, 51 and 103 genes were differentially expressed in the brain of male zebrafish (log_2>2, p<0.05) after exposure 235 ng/L and 291 µg/L diazepam, respectively. At 235 ng/L diazepam, 31 (61 %) genes were down-regulated, and 20 (39 %) up-regulated. Of the 103 genes differentially expressed at 292 µg/L diazepam 59 (57 %) genes were down-regulated, and 44 (43 %) were up-regulated. All 51 genes differentially expressed at the lower dose of 235 ng/L were also altered at 291 µg/L, and all of them were regulated in the same direction. Comparing our results with those obtained in goldfish (Popesku et al., 2008), it seems that different compounds acting as GABA agonists alter similar gene families. Similar to our study alterations in the aldehyde dehydrogenase family, GABA receptor subunits, hydroxysteroid dehydrogenase family, solute carrier, rev erb family, DnaJ-like genes and in the per3 gene have been observed. Diazepam is an allosteric modulator of the $GABA_A$ receptor, which is an inhibitory channel and can decrease the neuronal activity in its activated form (Sigel and Buhr, 1997). The $GABA_A$ receptor effects chloride permeability and hence, voltage dependent transmission of neural signals. Interference with this receptor will primarily affect neuronal activity, but not gene expression or cellular signal transduction pathways that may lead to changes in gene expression. Therefore, effects of diazepam on gene expression may be regarded as a secondary mode of action and side effects not directly related to the neurotoxic mode of action. On the other hand, the data may indicate hitherto unknown links and pathways related to the action of diazepam in the brain. In case of neuropharmaceuticals other than GABA agonists such as mianserin (van der Ven et al., 2006) alteration of other gene transcripts occur, as they act in a different manner.

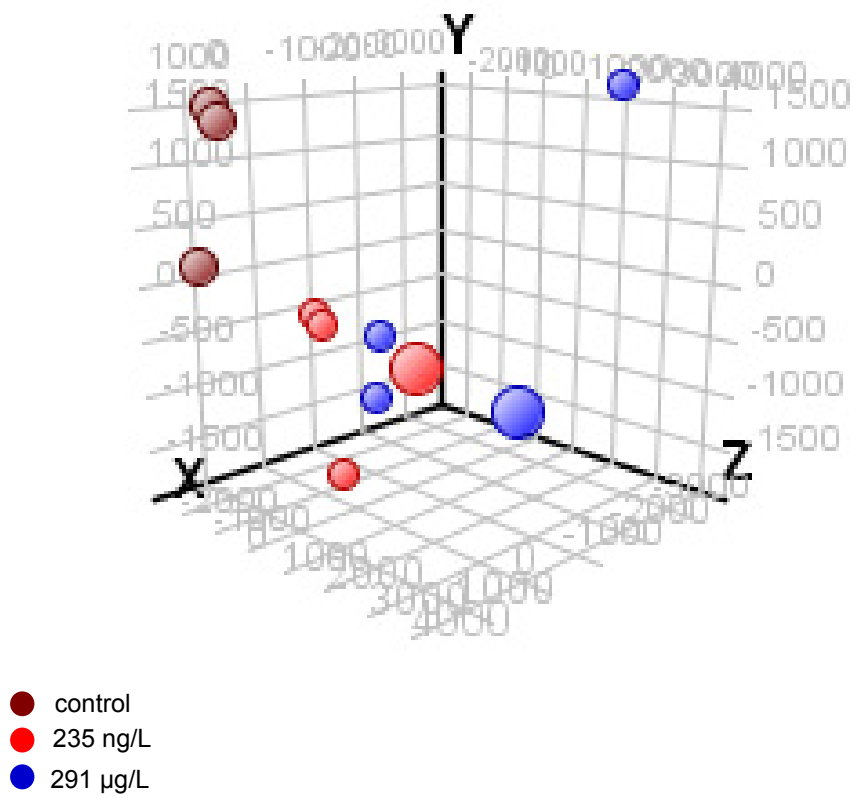

Figure 2

Three major dimensions of unsupervised PCA analysis. Brown dots are the control samples, red dots are the 235 ng/L diazepam treated samples, blue dots are the 291 µg/L diazepam treated samples. There exists a hyperplane that divides treated samples from untreated samples, so there is enough separability. However, samples do not group in concise clusters, so we have to consider all replicates within treatment.

Gene Ontology (GO) analysis was performed to identify functional groups of genes of interest. GO defines terms representing gene product properties and covers three domains namely cellular components, molecular functions and biological processes. At 235 ng/L diazepam, functionally identified genes fell into 57 different categories and at 291 µg/L diazepam into 40 categories. The following 8 categories shown in Figures 3A and 3B contain the highest percentage of differentially expressed genes: DNA photolyase activity, photoperiodism, IMP dehydrogenase activity, rhythmic process, circadian rhythm, GMP metabolic process, GMP biosynthetic process and response to temperature stimulus. In all of these 8 functional groups, a higher percentage of differentially expressed genes occurred at 291 µg/L diazepam as compared to 235 ng/L. Among those categories, alteration of genes involved in rhythmic processes, especially in the circadian rhythm, is of particular significance.

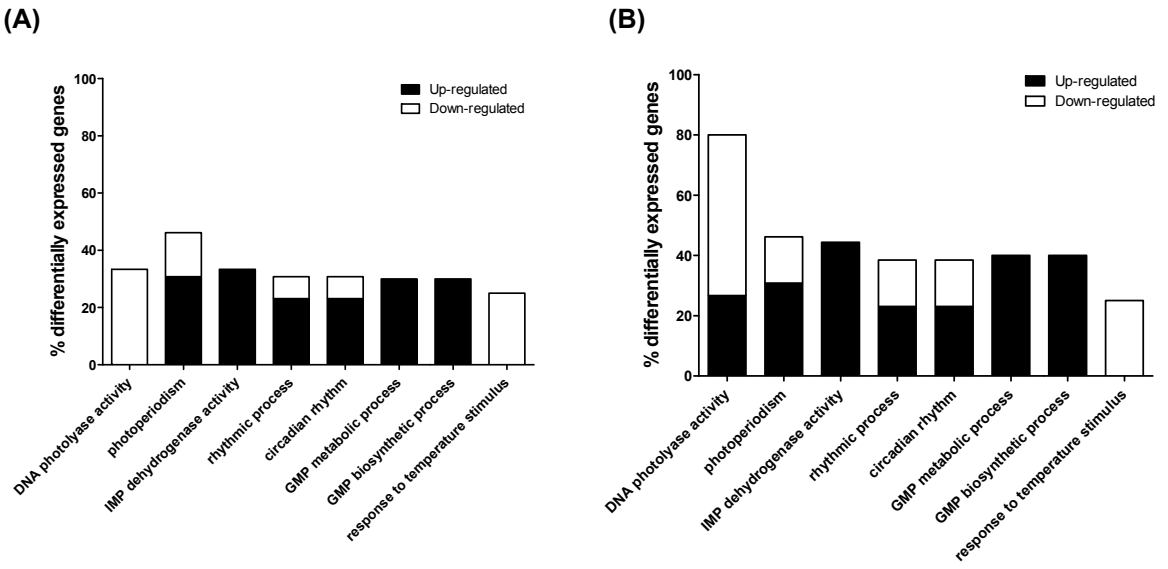

Figure 3

*Gene Ontology categories of differentially expressed genes (fold change (log_2) ≥ 2; $p < 0.05$) in zebrafish brain after exposure to **(A)** 235 ng/L and **(B)** 291 µg/L diazepam. Values are calculated as the percent of differentially expressed genes out of the total number of significantly altered genes.*

Through GABAeric influence on the hypothalamic suprachiastic nuclei (SCN), diazepam has been shown to affect the circadian rhythm in mammals (Carpentieri et al., 2006). The activation of the $GABA_A$ receptor can mediate phase-shifting in the circadian clock (Smith et al., 1989). In addition, $GABA_A$ receptor activation can lead to gene expression changes of circadian rhythm genes in mammals (Akiyama et al., 1999; Novak et al., 2006). However, the mechanism of this gene expression alteration is not fully understood. It is hypothesized that different stimuli - in our present study $GABA_A$ receptor activation by diazepam- can alter the phase of the circadian clock by increasing and decreasing the expression of different clock genes (Albrecht, 2002; Nishii et al., 2006). GABA receptors are highly conserved and occur also in fish (Carr and Chambers, 2001). Due to their high homology of up to 90% with the human GABA receptors, a similar mode of action in fish is expected (Christen et al., 2010). Indeed, diazepam showed a dose-dependent effect on the regulation of genes involved in the circadian rhythm (Table 4), including the down-regulation of period genes, which is in agreement with data in mice (Akiyama et al., 1999).

Table 4

Fold changes of selected genes involved in the circadian rhythm, whose expression was significantly altered in male zebrafish brain after 14 d exposure at 235 ng/L and 291 µg/L (fold change (log_2) ≥ 2; $p < 0.05$)

	Fold change (log_2)	
Gene name	**235 ng/L diazepam (measured conc.)**	**291 µg/L diazepam (measured conc.)**
aryl hydrocarbon receptor nuclear translocator-like 1a (***arntl1a***)	2.2	2.7
aryl hydrocarbon receptor nuclear translocator-like 1b (***arntl1b***)	2.4	3.2
aryl hydrocarbon receptor nuclear translocator-like 2 (***arntl2***)	2.7	5.6
basic helix-loop-helix domain containing, class B, 2 (***bhlhb2***)	-	- 3.1
basic helix-loop-helix domain containing, class B, 3 like (***bhlhb3l***)	- 2.3	- 3.5
cryptochrome 1b (***cry1b***)	- 2.5	- 3.2
cryptochrome 2b (***cry2b***)	1.7	3.3
cryptochrome 3 (***cry3***)	- 2.1	- 2.8
cryptochrome 4 (***cry4***)	1.5	2.1
cryptochrome 5 (***cry5***)	- 3.6	- 4.3
cryptochrome DASH (***cry-dash***)	- 3.2	- 3.9
nuclear factor, interleukin 3 regulated (***nfil3***)	2.1	2.5
nuclear receptor subfamily 1, group d, member 1 (***nr1d1***)	- 6.3	- 3.1
Rev-erbgamma A (***nr1d4a***)	1.6	4.4
Rev-erbgamma B (***nr1d4b***)	2.4	10.5
period homolog 1 (***per1***)	- 5.8	- 4.5
period homolog 3 (***per3***)	- 3.3	- 7.9
period homolog 4 (***per4***)	- 3.8	- 6.5
rar related orphan receptor C (***rorcb***)	4.8	7.4

Further experiments are needed to unravel the role of circadian rhythm gene expression alteration as a result of diazepam toxicity. Gene knock-downs of circadian rhythm genes could help to elucidate these observed gene expression changes. Additionally to the GO analysis, we performed a pathway analysis with MetaCore. The circadian rhythm is the most significantly altered pathway (Table 5).

In order to confirm microarray results, qRT-PCR was performed for selected genes. Five genes belonging to the circadian rhythm, *arntl2*, *cry2b*, *cry5*, *nr1d1* and *per1* were selected.

Additionally, the differential expression patterns of *arr3*, *gabrr1* and *hsd17b3* were verified as shown in Figure 4 and Table 6. The expression of a*rntl2* and *cry2b* showed a dose-dependent induction in adult fish, and a dose-dependent down-regulation in case of *cry5*, *nr1d1, per1, arr3, gabrr1* and *hsd17b3* (Figure 4, Table 6). The differential expression of these genes upon exposure of adult male zebrafish was confirmed in zebrafish eleuthero-embryos exposed to the same nominal concentrations of 273 ng/L and 273 µg/L diazepam (15 eleuthero-embryos pooled for each of the six replicates).

Table 5

Relevant pathways represented in MetaCore (FDR< 0.05).

Pathway	Pathway Group	Cell Process	p-Value	Gene
Circadian rhythm		Neurophysiological process	1.31E-10	6/47
Putative SUMO-1 pathway	Protein degradation	Proteolysis	2.83E-2	1/29
Urea cycle	Protein degradation		6.70E-2	1/70
Polyamine metabolism	Apoptosis		6.80E-2	1/71
GnRH signalling		Reproduction	6.89E-2	1/72
(L)-Arginine metabolism	Aminoacid metabolism and its regulation		7.26E-2	1/76
GTP-XTP metabolism	Nucleotide metabolism and its regulation		8.54E-2	1/90
Arginine metabolism	Aminoacid metabolism and its regulation		9.18E-2	1/97

The same genes were up- and down-regulated, respectively. These changes in transcripts were particularly found in genes involved in the circadian rhythm. It is known that the circadian clock becomes functional already on the first day of development in zebrafish embryos (Dekens and Whitmore, 2008). Although the same trends in the expression pattern occurred in the microarray and qRT-PCR data of adult fish and eleuthero-embryos for most of the genes, statistical significance and absolute fold-change differed between the methods and developmental stages. Exceptions were g*abrr1* and *hsd17b3*, which presented an up-regulation in eleuthero-embryos, but a down-regulation in adults (both microarray and qRT-PCR data).The alteration of a nearly identical set of genes at both concentrations in adults and eleuthero-embryos demonstrates that the effect of diazepam is consistent in both life stages, showing effects on gene expression already at nominal concentrations of 273 ng/L.

The almost identical pattern of gene expression profiles also indicates that diazepam acts through a neuropharmacological mode of action in fish on a molecular level, similar to that in mammals. This supports the notion that pharmaceuticals should be assessed for potential environmental risks through their mode of action.

Table 6

Fold changes of selected genes differentially regulated in zebrafish brain determined by microarray and qRT-PCR in adult zebrafish brain as well as qRT-PCR determination in zebrafish eleuthero-embryos after exposure to low concentration (235 ng/L) and high concentration (291 µg/L) of diazepam (adults) and nominal 273 ng/L and 273 µg/L (eleuthero-embryos), respectively. Values are expressed as average fold change (log_2).

	Fold Change (log_2)						
Gene name	**low concentration diazepam**				**high concentration diazepam**		
	Array (adult)	**qRT-PCR (adult)**	**qRT-PCR (eleuthero-embryos)**		**Array (adult)**	**qRT-PCR (adult)**	**qRT-PCR (eleuthero-embryos)**
aryl hydrocarbon receptor nuclear translocator-like 2 (***arntl2***)	2.7	1.5*	0.8*		5.6	2.8*	2.0*
cryptochrome 2b (***cry2b***)	1.7	0.9*	1.6*		3.3	1.8*	2.7*
cryptochrome 5 (***cry5***)	- 3.6	-1.8*	-0.8*		- 4.3	-1.9*	-1.3*
nuclear receptor subfamily 1, group d, member 1 (***nr1d1***)	- 6.3	-2.7*	-1.0		- 3.1	-1.7*	-1.3
period homolog 1 (***per1***)	- 5.8	-2.5*	-2.0*		- 4.5	-2.2*	-3.6*
arrestin 3, retinal (X-arrestin) (***arr3***)	- 3.8	-0.7	-0.5		- 4.4	-1.7*	-0.7
gamma-aminobutyric acid (GABA) receptor, rho 1 (***gabrr1***)	-2	-1.2*	0.2		- 4.1	-2.3*	0.04
hydroxysteroid (17-beta) dehydrogenase 3 (***hsd17b3***)	- 1.6	-0.3	0.2		- 2.3	-1.1*	0.6

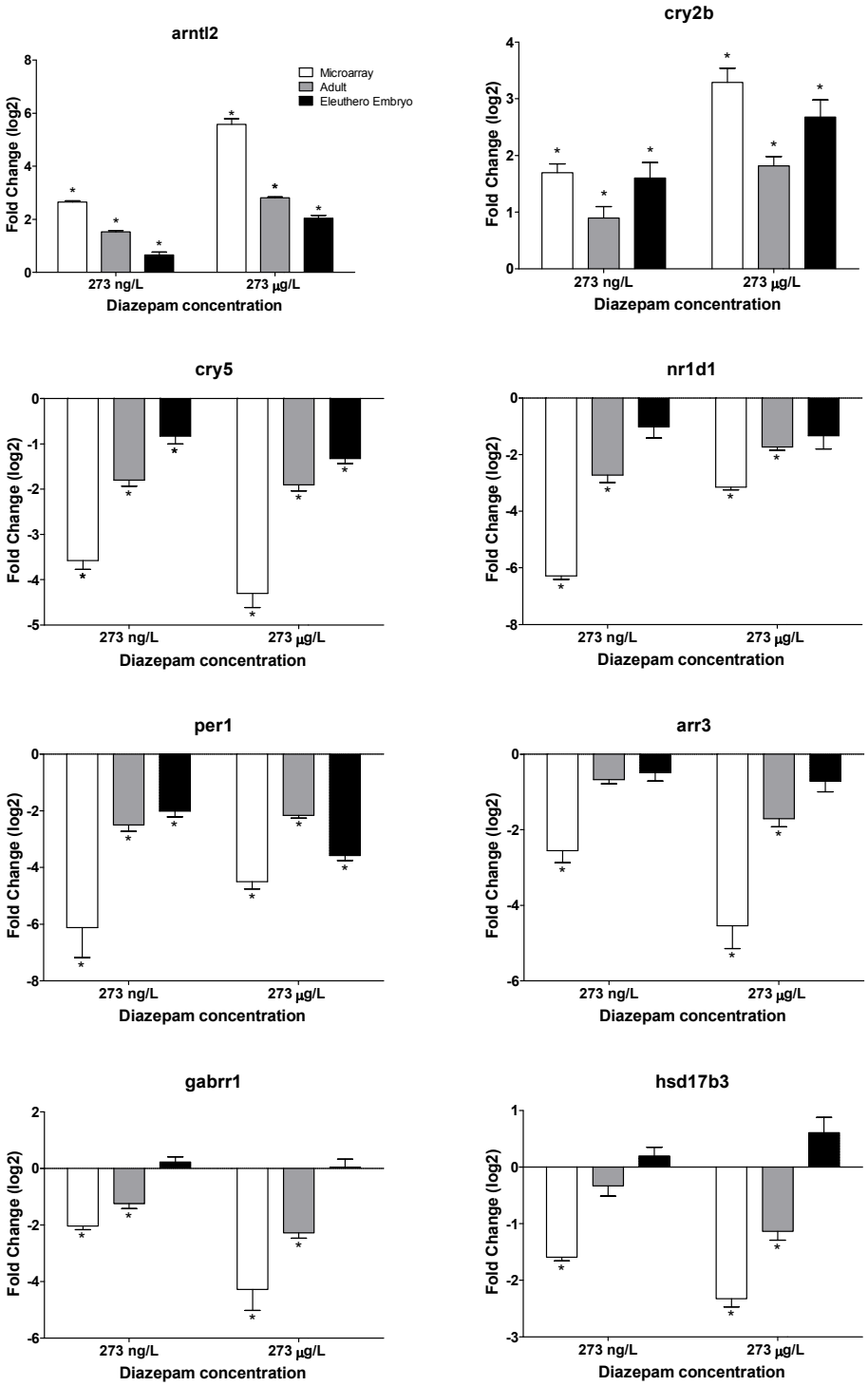

Figure 4

Comparison of gene expression in zebrafish brain determined by microarray (n = 4 replicates, 15 male fish pooled, white bars), qRT-PCR (n = 5, 15 male fish pooled, grey bars) determined in zebrafish brain and qRT-PCR measured in zebrafish eleuthero-embryos (n = 6, 15 eleuthero-embryos pooled, black bars) after exposure to nominal 273 ng/L and 273 µg/L diazepam, respectively. Values are expressed as average fold change (log_2) with standard error compared to control animals as indicated for selected genes. Asterisks, statistically significant difference to control ($p < 0.05$).

3.3.2 Effects of diazepam on the circadian rhythm and locomotor activity

In mammals, the circadian rhythm controls a diversity of behavioral and physiological rhythmic processes such as regulation of sleep wakefulness, secretion of hormones and locomotor activity (Hastings, 1997). Therefore, a second set of experiments was performed to evaluate whether changes found in the gene expression pattern of circadian genes in the brain were paralleled by changes in the locomotor activity of adult zebrafish and eleuthero-embryos. The behavioral experiments revealed that adult males did not show statistically significant differences to controls in their locomotor activity, both after 3 and 14 d of exposure to diazepam (Figure 5). We chose two time-points (3 and 14 d) to test for possible tolerance to diazepam, as found in *Gambusia holbrooki* (Nunes et al., 2008). The lack of significant changes in locomotion in adult fish may be interpreted in a way that the gene alterations were not propagated to significant behavioral changes, or alternatively as rapid tolerance against diazepam exposure. However, there is a dose-dependent trend to a more locally restricted swimming behavior after the 14-d exposure, which is interpreted as an indication of the known sedative effect of diazepam (Figure 5).

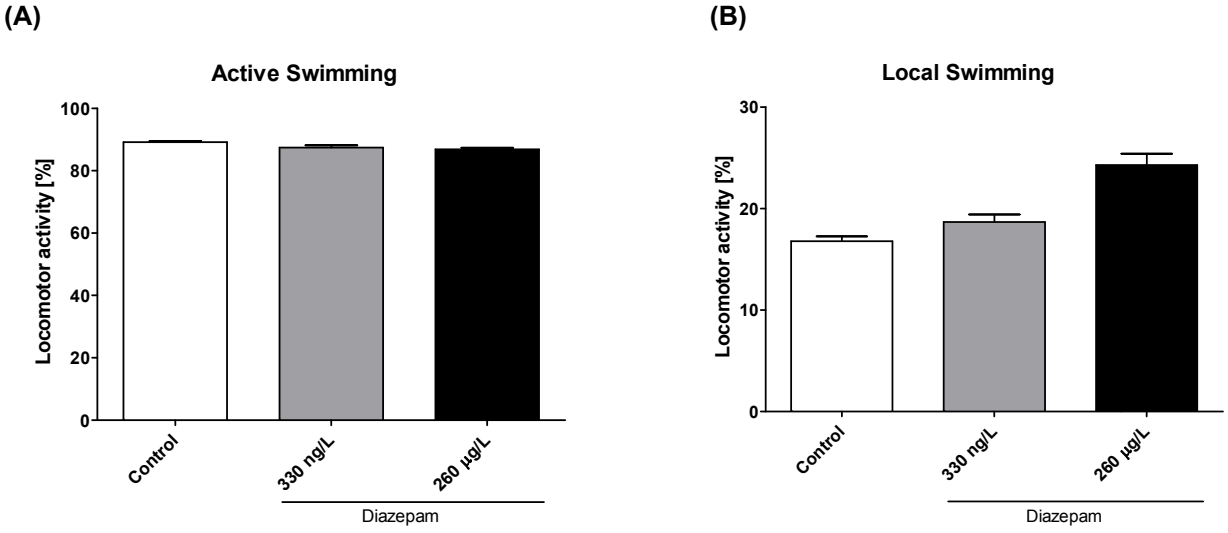

Figure 5

*Comparison of the locomotor activity (percent of total time spent in locomotion during 2 h) of adult D. rerio exposed for 14 d to 330 ng/L and 260 µg/L diazepam and held in water (control). (**A**) Active swimming behaviour showing a slight decrease in diazepam treated fish. (**B**) Locally restricted swimming and ventilation showing a slight increase in diazepam treated fish. Significant differences between treatments: *p < 0.05.*

In eleuthero-embryos the locomotor activity at nominal concentration of 273 µg/L diazepam was distinctly different from that of control eleuthero-embryos. The diazepam-exposed eleuthero-embryos featured higher activity with less pauses. Movement patterns of controls (Figure 6A) differed from that of diazepam-exposed eleuthero-embryos (Figure 6B). The

locomotor activity was significantly increased at 273 µg/L diazepam compared to eleuthero-embryos exposed to 273 ng/L and the controls, which did not differ from each other (Figure 7). This indicates that eleuthero-embryos seem to react more sensitive in their behavior than adults and that diazepam provoked a “paradoxical reaction”, e.g. restlessness instead of sedation. This observation was also made in children, who became hyperactive after administration of diazepam (Saias and Gallarda, 2008). However, it was observed that zebrafish eleuthero-embryos locomotor activity was reduced when treated with 1 µM (284.7 µg/L) diazepam (Zhdanova et al., 2001). The discrepancy to our observation may be explained that the locomotor activity was determined in the dark, whereas we measured it in the light.

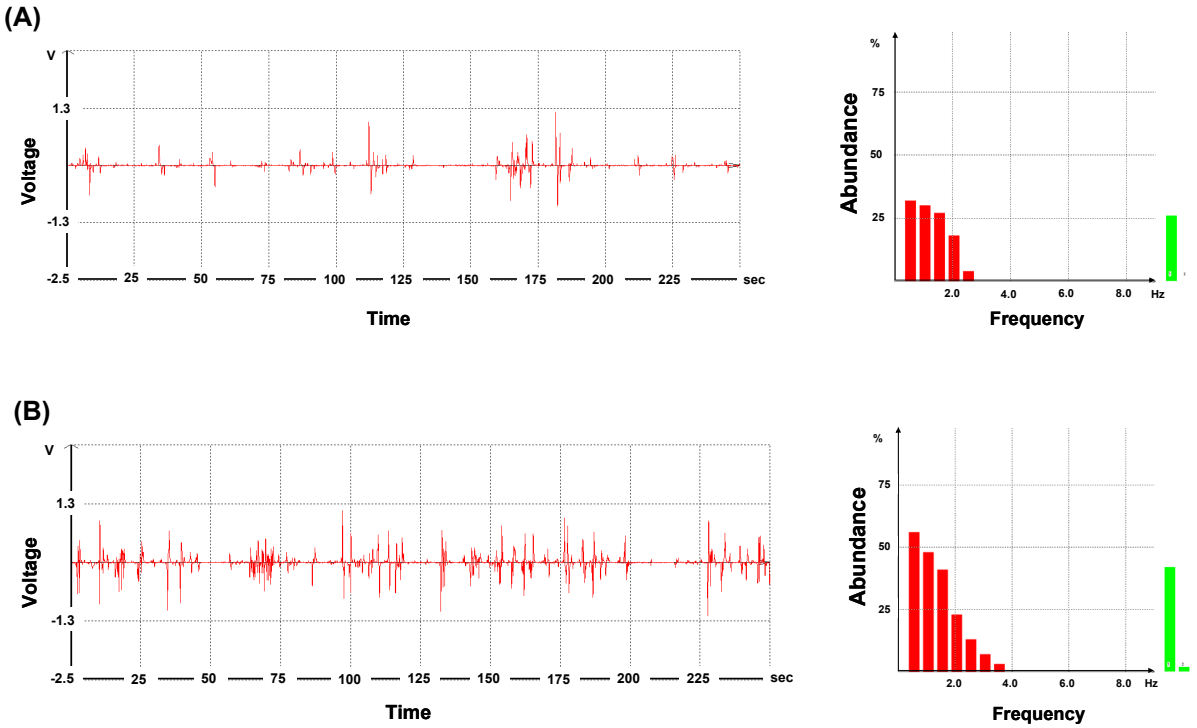

Figure 6

Typical examples of spontaneous locomotor movement patterns. Movement pattern (left; amplitude (V) vs. time (s) recorded over 250 s) and fast Fourier transformation (FFT) histogram (right; activity in % of the time (250 s)). ***(A)*** *Control D. rerio eleuthero-embryos.* ***(B)*** *D. rerio eleuthero-embryos treated with 273 µg/L diazepam showing an increased locomotor activity. The red bars in the FFT histogram (right) indicate the different frequencies of locomotion and ventilation. Signals for locomotion were in the range of 0.5 to 2.5 Hz (green bar left), and frequencies for ventilation lay above 2.5 Hz (green bar right).*

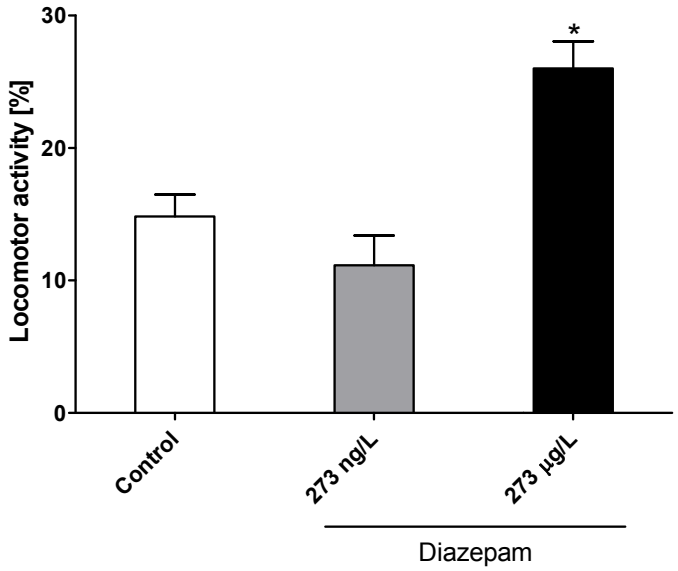

Figure 7

Comparison of the locomotor activity (percent of total time spent in locomotion during 2 h) of D. rerio eleuthero-embryos exposed for up to 3 d after hatching to nominal concentration of 273 ng/L and 273 µg/L or water control. Asterisks, significant differences between treatments ($p < 0.05$).

3.3.3 Effects of diazepam on *hsd17β3* expression and testis histology (unpublished data)

In mammals testosterone formation and inactivation are performed by different members of the hsd17β enzymes. Zebrafish hsd17β3 catalyzes the conversion of androstenedione to testosterone *in vitro* (Mindnich et al., 2005). In our study, we demonstrated that *hsd17β3* is significantly down-regulated after exposure to diazepam (Figure 4, Table 6). In order to assess potential physiological and histological effects of this down-regulated gene, the testes of exposed adult zebrafish were analyzed histologically. In males exposed to 235 ng/L and 291 µg/L diazepam, we observed no statistically significant alterations in the frequency of different development stages of sperms (spermatogonia, spermatocytes and spermatides) in the seminiferous tubuli. However, a tendency to an altered pattern of the different stages of sperms in the seminiferous tubuli was observed, which was characterized by the occurrence of more interstitial tissue (Figure 8). In the interstitial tissue Leydig cells, harboring a GABAergic system, are mainly found. In humans it was shown that GABA and benzodiazepines are able to stimulate Leydig cell proliferation and regulate the steroid synthesis by Leydig cells via the $GABA_A$ receptor (Geigerseder et al., 2004). Our results are in agreement with this observation, but are in contrast to results obtained in rats, where shrinkage of seminiferous tubules and a significant reduction of spermatocytes occurred. Additionally, the Leydig cells were small and there were wide gaps between the spermatocytes (Siddiqui et al., 2008). In our study using microarrays, we identified mainly the

alteration of genes involved in regulation of the circadian rhythm as a significant mode of action of diazepam in zebrafish. Nevertheless, attention should also be placed on other mechanisms such as alteration of steroidogenesis.

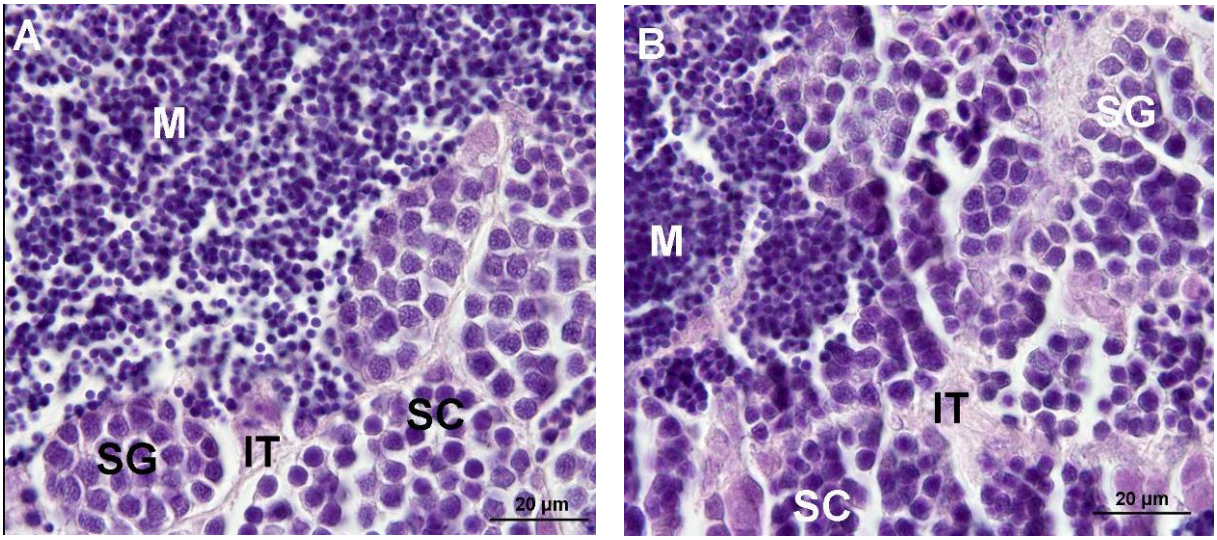

Figure 8

*Histology of zebrafish testes; of (**A**) control male and (**B**) male exposed to 291 µg/L diazepam. SG= spermatogonia; SC= spermatocytes; M= mature spermatids; IT = interstitial tissue.*

In our study we show that the response of zebrafish to diazepam is similar to the human response. Significant changes in gene expression of circadian rhythm, which is also altered in mammals, occurred at low concentrations of 235 ng/L diazepam. This demonstrates that alterations in gene expression are may be more sensitive than behavioral, histological or other toxicological effects (Straub, 2008), although no mechanistic support for the potential role of the circadian rhythm genes in mediating neurotoxic effects is provided or yet known. The lack of behavioral and physiological changes at the low concentration, however, raises the question when gene expression changes propagate to toxicological relevant measures such as reduction of the ability to find food, to reproduce or to escape from predators. In contrast a positive correlation between alteration in gene expression and behavior occurred at the higher diazepam concentration; therefore, the gene expression pattern is interpreted as indicative for effects at the organism level.

In conclusion the present study confirms that the toxicogenomic approach provides important data to identify and characterize molecular effects and to elucidate potential modes of action of a pharmaceutical found in the environment. This allows for establishing new biomarkers, not regularly assessed in routine ecotoxicological studies. However, molecular studies should be paralleled with ecotoxicological investigations of known ecological relevance.

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Chapter 4

Effects of the protein kinase inhibitor PKC412 on gene expression and link to physiological effects in zebrafish *Danio rerio* eleuthero-embryos

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Abstract

To identify molecular effects of the antineoplastic agent PKC412 (Midostaurin), we applied gene expression profiling in zebrafish using whole genome microarrays. Behavioral, developmental and physiological effects were investigated in order to analyze for correlations between altered gene-expression profiles with effects on development and physiology. Zebrafish blastula-stage embryos were exposed for 6 days post fertilization (dpf) to nominal levels of 2 µg/L and 40 µg/L PKC412. Among the 259 and 511 altered transcripts at both concentrations, respectively, the expressions of genes involved in the circadian rhythm were further investigated. Alteration of swimming behavior was not observed. Pathways of interest affected by PKC412 were angiogenesis, apoptosis, DNA damage response and response to oxidative stress. Angiogenesis was analyzed in double transgenic zebrafish embryos Tg(fli1a:EGFP)y1;Tg(gata1:dsRed)sd2; no major defects were induced by PKC412 treatment at both concentrations. Apoptosis occurred in olfactory placodes of embryos exposed to 40 µg/L, and DNA damage was induced at both PKC412 concentrations. However, there were no significant effects on reactive oxygen species formation. This study leads to the conclusion that PKC412-induced alterations of gene transcripts are partly paralleled by physiological effects at high, but not at low PKC412 concentrations expected to be of environmental relevance.

Key words: PKC412, *Danio rerio*, transgenic zebrafish, microarray, angiogenesis, apoptosis

4.1 Introduction

Antineoplastic agents have widespread use in cancer treatment. They include different classes, such as antibodies, protein kinase inhibitors (such as PKC412), topoisomerase I and II inhibitors, antimetabolites, alkylating agents and others (Chabner et al., 2006). Antineoplastic agents are mainly found in the ng/L to the lower µg/L range in effluents (Monteiro and Boxall, 2010). For example ifosfamide was found in Germany up to a concentration of 2.9 µg/L (Ternes, 1998).

As many pharmacological targets are evolutionary conserved, antineoplastic agents are supposed to have similar effects in aquatic organisms as in humans (Jones et al., 2007; Gunnarsson et al., 2008) Therefore, environmental exposure may pose a risk for negative effects on non-target species, due to the mutagenic and cancerogenic properties of such compounds.

A newly developed antineoplastic agent is midostaurin (PKC412, $\log K_{OW}$ = 4.26; Novartis Pharma AG, internal data) which belongs to the family of indolocarbazoles and is a selective inhibitor of several isoforms of protein kinase C in humans (Karaman et al., 2008). It is a sugar ring variant of staurosporine which was originally isolated from *Streptomyces staurosporeus* (Takahashi et al., 1989). PKC412 inhibits a large variety of tyrosine kinases including FLT3 (FMS-like tyrosine kinase), PDGF (platelet derived growth factor) receptors and c-kit (stem cell factor) receptor (Fabbro et al., 2000). PKC412 has been developed as a therapeutic agent against acute myeloid leukemia (AML), because of its ability to inhibit growth, angiogenesis and P-glycoprotein mediated multidrug resistance in tumor cells (Fabbro et al., 2000). In addition, PKC412 affects other cellular processes such as immune responses or neuronal functions, as shown by effective inhibition of human T-cell activation, proliferation and TNFα production (Si et al., 2005).

Only very few data are available on the toxicity of PKC412 to organisms that may be exposed via wastewater. Exposure of zebrafish blastula-stage embryos to 100 nM (57 µg/L) PKC412 resulted in a curved body axis (Chan et al., 2002). Recent data indicates that the no observed effect concentration (NOEC) of PKC412 was 14 µg/L in a *Danio rerio* early life stage test, and the lowest observed effect concentration (LOEC) for mortality was 43 µg/L. The 96 h LC_{50} in adult *D. rerio* was 25 µg/L and the NOEC 19 µg/L (Novartis Pharma AG, internal data). The estimated predicted environmental concentration (PEC) is 1.5 µg/L (Novartis Pharma AG, internal data).

Currently there is a lack of chronic toxicity studies with focus on the modes of action on this pharmaceutical in vertebrates and invertebrates. Furthermore, ecological risk assessments of PKC412 within the framework of EMEA (2006), in particular at environmentally realistic concentrations in aquatic organisms, are not publicly available. Ecotoxicological tests used for risk assessments are often not sensitive enough to identify subtle adverse effects of pharmaceuticals. They may be more accurately determined by focusing on the modes of action (Fent et al., 2006; Runnalls et al., 2007). Therefore, potential environmental consequences of PKC412 are unknown. In detail, data on the gene expression profile and their correlation to environmentally relevant endpoints such as behavior and mortality are missing.

In our present study, we aim at determining the modes of action of this novel antineoplastic agent in zebrafish embryos to clarify its potential molecular effects by analyzing the global gene expression pattern. The toxicogenomics approach allows to identify several thousands of genes and the corresponding expression profiles upon exposure, which will assist in the elucidation of the molecular effects and the compound's modes of action (Robbens et al., 2007). We analyze effects at low concentrations in zebrafish embryos and compare the effects on the transcriptional level with effects on mortality and physiological outcomes. We hypothesize that the response of zebrafish to PKC412 exposure is similar to the human response, as their targets, the protein kinases, are evolutionary conserved. Therefore, we searched for alterations in gene expression patterns associated with cellular signaling (e.g. angiogenesis, apoptosis), and oxidative stress. We also hypothesized that alterations in gene expression - because of multiple endpoints and mechanistic information - are more sensitive than physiological or morphological parameters (although they are perhaps less ecologically relevant), but that the observed molecular effects correlate with and propagate to higher levels of the biological organization. In addition, gene expression analysis may also reveal unknown regulatory mechanisms in fish not directly related to the modes of action of PKC412 in humans.

4.2 Materials and Methods

4.2.1 Chemicals

PKC412 (≥ 99%, MW: 570.6, $\log K_{OW}$ = 4.26) was kindly provided by Novartis Pharma AG (Basel Switzerland). Acetonitrile was purchased from Brunschwig (Basel, Switzerland) and methanol was from Stehelin (Basel, Switzerland). Ammonium formiate, dimethyl sulfoxide (DMSO), formic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), $MgCl_2$, NaCl, sucrose, Triton X-100, H_2O_2, 4',6-diamidino-2-phenylindole (DAPI) and

dichlorofluorescein-diacetate (DCFH-DA) were obtained from Sigma Aldrich (Buchs, Switzerland). 4-2-Aminoethyl-benzensulfonyl fluoride hydrochloride (AEBSF), ethylene diamine tetraacetic acid (EDTA), low melting agarose and Tris-HCl were purchased from AppliChem.GmbH (Darmstadt, Germany). Roti®-Histofix 10% was purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). PBS was obtained from F. Hoffmann-La Roche Ltd (Basel, Switzerland)

4.2.2 Exposure experiment

For the microarray, behavioral and toxicity studies, freshly fertilized zebrafish embryos were obtained from Harlan Laboratories Ltd. (Itingen, Switzerland). Embryos were transferred to the laboratory and examined under a stereomicroscope. All embryos used in the experiments were at blastula stage (Kimmel et al., 1995). They were placed in 750 mL covered glass beakers of reconstituted water (total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 µS/cm) and the appropriate concentration of PKC412 or DMSO (0.01 %, solvent control). The water temperature was held constant at 27±1 °C with the photoperiod set at 16:8 h light/dark.

The semi-static exposure setup consisted of six replicates of water control, solvent control (0.01 % DMSO) and two PKC412 doses. A total of 100 fertilized eggs per replicate (n = 6) were exposed up to 6 days post fertilization (dpf) to nominal concentrations of 2 µg/L and 40 µg/L PKC412, respectively. This exposure duration seemed to be appropriate as lethal effects occurred already after 3 dpf in the ELS test. Every 24 h, lethal and sublethal effects were evaluated, dead embryos or eleuthero-embryos were removed and the water was changed. The quality of the exposure water was continuously monitored by oxygen concentration determination (>70%), the pH value (6.7-7.2) and the temperature (27±1 °C). At the end of exposure, eleuthero-embryos were anaesthetized in a clove oil solution (Fluka AG, Buchs, Switzerland). A total of 80 eleuthero-embryos per replicate were pooled in RNAlater for microarray and qRT-PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland).

4.2.3 Chemical analysis

To determine actual exposure concentrations, 10-mL aliquots of exposure water were taken during the experiment for PKC412 analysis. Water samples of each treatment group were taken at the beginning (0 h) and prior to full water renewal (24 h). This was done three times on different days from different randomly selected replicate tanks. Acetonitrile (2 mL) was added to the water samples for LC-MS analysis preparation, and then stored at -20 °C until analysis. The chemical analysis was performed separately for the microarray experiment, the

behavioral experiment and additional experiments (TUNEL assay, comet assay, ROS assay).

PKC412 concentrations were determined by LC-MS. The chromatographic separation was achieved by using an Inertsil ODS-3 column (2.1 mm x 50 mm; 3 µm particle size) from Ercatech AG (Bern, Switzerland) at a column temperature of 30° C. For the analysis a binary gradient mixture containing eluent A (0.1% (v/v) in formic acid in water:acetonitrile (95:5)) and eluent B (0.1% (v/v) in formic acid in water:acetonitrile (5:95)) was used at a flow rate of 0.4 mL/min. The gradient started with a mixture of 60% eluent A and 40% eluent B and increased to 100% eluent B after 4 min. The conditions were held for 1 min and then the system was set back to the initial conditions. The column was re-equilibrated for 2 min before the next injection. 5 µL of the samples were injected. The retention time for PKC412 was 3.3 min. Data was then quantified using DataAnalysis for 6300 Series Ion Trap LC/MC version 3.4. Quantification of PKC412 was based on an external calibration curve.

4.2.4 RNA isolation, array hybridization and sample selection

Total RNA was extracted from zebrafish eleuthero-embryos using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). Total RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 UV-VIS Spectrophotometer at 260 nm. The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing a 260/280 nm ratio between 1.8-2.1, a 28S/18S ratio between 1.5-2 and an RNA integrity number (RIN) > 8 were processed further. A total of 16 arrays (Agilent 4 × 44 K Zebrafish microarray) were used, including four for the water control group, four for the solvent control group, four for the 2 µg/L and four for the 40 µg/L PKC412 dose group. Total RNA samples (600 ng) were reverse-transcribed into double-strand cDNA in the presence of RNA poly-A controls with the Agilent One-Color RNA Spike-In Kit. Cy3 labeling and hybridization were performed according to the manufacturer's manual.

After reverse-transcription of RNA into double-stranded cDNA, double-strand cDNA was *in vitro* transcribed into cRNA in the presence of Cy3 labeled nucleotides using a Low RNA Input Linear Amp Kit +Cy dye (Agilent Technologies, Basel, Switzerland), performed at the Functional Genomic Centre (ETHZ and University of Zürich, Switzerland). The Cy3-labeled cRNA was purified using an RNeasy mini kit (Qiagen, Basel, Switzerland), and quality and quantity was determined using a NanoDrop ND-1000 UV-VIS Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Only cRNA samples with a total cRNA yield higher than 2 µg and a dye incorporation rate between 9 pmol/µg and 20 pmol/µg were used for

hybridization. Cy-3-labeled cRNA samples (1.65 µg) were mixed with Agilent blocking solution, subsequently fragmented randomly to 100-200 bp at 65 °C with fragmentation buffer and resuspended in hybridization buffer as provided by the gene expression hybridization Kit (Agilent Technologies). Target cRNA samples (100 µL) were hybridized to the Agilent Zebrafish 4x44K Gene Expression Microarray for 17 h at 65 °C. The hybridized arrays were then washed using Agilent GE wash buffers 1 and 2 according to the manufacturer's instructions and scanned by an Agilent Microarray Scanner (Agilent p/n G2565BA) at 5 µm resolution with the green photomultiplier tube set to 100% and a scan area of 61 x 21.6 mm. Image generation and feature extraction was performed using the Agilent Feature Extraction (FE) software version 9.5.3. Quality control was additionally considered before performing the statistical analysis. These included array hybridization pattern inspection: absence of scratches, bubbles, areas of non-hybridization, proper grid alignment, spike performance in controls with a linear dynamic range of 5 orders of magnitude and the number of green-feature non-uniformity outliers which should be below 100 for all samples.

4.2.5 qRT-PCR analysis

1 µg of total RNA of the microarray experiment template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min.

The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). Eight differentially expressed genes of interest (ATP-binding cassette, sub-family C (*abcc4*), cryptochrome 5 (*cry5*), N-myc downstream regulated gene 1, like (*ndrg1l*), opsin 1 (cone pigments), medium-wave-sensitive, 2 (*opn1mw2*), 3-phosphoinositide dependent protein kinase-1 (*pdpk1*), period homolog 1 (*per1*), similar to son of sevenless homolog 2 (*sos*), xeroderma pigmentosum, complementation group C (*xpc*)) were selected for confirmation of microarray results using qRT-PCR. Gene-specific primers were designed based on published zebrafish sequences (Table 1).

The following PCR reaction profile was used: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec, and 59 °C for 60 sec followed by a melting curve analysis post run.

The delta CT value was derived by subtracting the threshold cycle (CT) value for the housekeeping gene ribosomal protein L13 α (*RpL13α*), which served as an internal control, from the CT value of the target gene, respectively. All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). The mRNA expression level of the different genes was expressed as fold-increase according to the formula:

$2^{\Delta CT(\text{untreated sample})- \Delta CT(\text{treated sample})}$

Table 1

Primer sequences for quantitative real-time PCR analysis

Probe ID	Gene	Sequence Accession #	dir	Sequence	Amplicon size
-	RpL13α	NM_212784	fw rv	agc tca aga tgg caa cac ag aag ttc ttc tcg tcc tcc	100 bp
A_15_P104624	abcc4	NM_001007038	fw rv	ctg gaa aca cga ctc agc aa gct cac cag agc att gaa ca	122 bp
A_15_P103946	cry5	NM_131788.1	fw rv	cat gga gag aac gaa ctg gg gtg cag aca agc agc cga ac	115 bp
A_15_P196411	ndrg1l	NM_200692.1	fw rv	agc gtc ttt gag ctg gac at tgg aag gtc agg atg gta gg	113 bp
A_15_P544897	opn1mw2	NM_182891.2	fw rv	cca tgg cag ttt aag gca ct ttt tgt gct gag ctg tga cc	100 bp
A_15_P112731	pdpk1	NM_001077344.1	fw rv	cgt tta gag ctg gga acg ag cgc tgg acc aga tct tta gc	103 bp
A_15_P236266	per1	NM_001030183.1	fw rv	atg cgt gca aga agt ggt g acg tcc tca ttt agc gga ctc	131 bp
A_15_P197856	sos	XM_685079.2	fw rv	ctg ccc tca ctt ctc acc tc cac tgg tcc aca cca aac ac	109 bp
A_15_P134231	xpc	NM_001045210.1	fw rv	aag aag tcg gca gtg agg aa gca tat ttt cac ggc tcc at	101 bp

abcc4 (ATP-binding cassette, sub-family C), ***cry5*** (Cryptochrome 5), ***ndrg1l*** (N-myc downstream regulated gene 1, like*)*, ***opn1mw2*** *(*opsin 1 (cone pigments), medium-wave-sensitive, 2), ***pdpk1*** (3-phosphoinositide dependent protein kinase-1), ***per1*** (period homolog 1), ***sos*** (similar to son of sevenless homolog 2), ***xpc*** (xeroderma pigmentosum, complementation group C)

4.2.6 Measurement of locomotor activity

Multispecies Freshwater Biomonitor®. Effects of PKC412 on locomotor activity were measured in zebrafish eleuthero-embryos. Their locomotor activity was observed using a flow-through test chamber with quadrupole impedance conversion as measuring device, connected to a measuring unit and personal computer with data analysis software (Gerhardt

et al., 1994). Measurement chambers, made of an acryl glass cylinder sealable on both ends, with a size of 4 cm in length and a diameter of 2 cm allowed free movement of the eleuthero-embryos during measurement.

For locomotor activity measurements, embryos in the blastula stage were exposed to 2.1 µg/L and 31 µg/L PKC412, respectively, for 6 dpf as described above. Locomotor activities of hatched eleuthero-embryos were assessed 6 dpf for 20 animals per PKC412 dose and the appropriate controls, respectively, as described above. Following an acclimation phase of 10 min, measurements were started and behavior was monitored with a recording duration lasting 4 min for a period of 2 h with intervals of 10 min each. For locomotor activity measurements, means of locomotor activities (% time spent on locomotion) for each individual were calculated for 2 h time period.

4.2.7 Angiogenesis

Double transgenic zebrafish embryos (Tg(fli1a:EGFP)y1;Tg(gata1:dsRed)sd2) (Lawson and Weinstein, 2002; Traver et al., 2003) were exposed to 2 and 40 µg/L PKC412 and held in water and DMSO control water (50 embryos each) for 6 dpf as described above. Embryos were monitored for morphological defects using a fluorescent binocular at 24 hpf, 48 hpf and 6 dpf. Formation of blood vessels was observed with transgenic expression of enhanced-green-fluorescent-protein (EGFP) in endothelial cells and DsRed (red fluorescent protein from *Discosoma sp.*) expression in blood cells. Transgenic fish were kept heterozygotic, therefore only half of the progeny is double transgenic and these were analyzed in detail. Representative embryos taken from treated and control groups were imaged using Leica TCS SP5 Confocal Microscopy System.

4.2.8 Apoptotis assay

Apoptotic cells of eleuthero-embryos were determined by the TUNEL assay. Eleuthero-embryos were exposed 6 dpf as described above. Embryos exposed to 1% H_2O_2 served as a positive control. After 6 dpf, the eleuthero-embryos were fixed in 4% Roti®-Histofix at 4° C for 24 h. Thereafter, the embryos were rehydrated through a series of methanol (100%, 90%, 70% and 50%). The TUNEL assay (Roche "*In situ* Cell Death Detection Kit, TMR red") was performed as described in the manufacturer's manual. In brief, single eleuthero-embryos (n = 3 per replicate) were permeabilized in phosphate buffered saline containing 0.1% Triton X-100 (PBST) for 8 min. Thereafter, they were washed twice with PBS and then labeled for 60 min at 37 °C. The embryos were then embedded in agarose and analyzed under the

fluorescent microscope at excitation and emission wavelength of 360-370 nm and 420 nm, respectively.

4.2.9 Alkaline comet assay

This assay allows detecting DNA breakages induced by genotoxic agents. The comet assay was performed as described (F. Duong, University Hospital Basel, Switzerland, personal communication). Briefly, embryos were exposed to PKC412 as described above for 6 dpf. As a positive control, embryos were exposed to 1% H_2O_2 for 10 min prior to anaestization. A pool of 8 eleuthero-embryos per replicate (n = 4) was macerated in 1 mL PBS containing 20 mM EDTA for 5 min. The supernatant was removed and the embryos were shred in 500 µL PBS containing 20 mM EDTA for 10 min. The remaining tissue was sedimented for 2 min and the supernatant was embedded in agarose on a microscope slide. Cells were then lysed at 4 °C for 1 h with lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 10, 1% Triton X-100) and electrophoresed for 5 min at 25 V. Damaged DNA fragments migrate faster in the electric field than intact DNA which can be visualised after staining with 4',6-diamidino-2-phenylindole (DAPI). Cells (24 cells per replicate) were observed under the fluorescence microscope and the length of comets, % DNA in tail, tail moment and olive tail moment were calculated using CometScore 1.5 (TriTek$_{TM}$, Sumerduck, VA, USA).

4.2.10 ROS measurements

In this assay, the non-fluorescent probe DCFH-DA is oxidized into the highly fluorescent 2,7-dichlorofluorescein in the presence of reactive oxygen species (ROS). ROS measurements were conducted according to (Deng et al., 2009). In brief, 8 pre-exposed eleuthero-embryos (pre-exposure for 6 dpf as described for the microarray experiment) were washed with cold PBS and then homogenized in cold lysis buffer containing 0.32 mM sucrose, 20 mM HEPES, 1mM $MgCl_2$ and 0.5 mM AEBSF, pH 7.4. As a positive control, 8 eleuthero-embryos were exposed for 10 min to water containing 1% H_2O_2. Thereafter the homogenate was centrifuged at 15'000g at 4 °C for 20 min and the supernatant was discharged. The pellet was then resuspended in PBS and 20 µL were added to a 96-well plate and incubated for 5 min at room temperature. Thereafter, 100 µL of PBS and 8.3 µL of DCFH-DA (stock solution in DMSO, 10 mg/mL) were added to each well. The plate was incubated at 37 °C for 30 min and fluorescence measurements were made with excitation at 485 nm and emission at 530 nm, respectively.

4.2.11 Data analysis and statistics

Raw microarray data was analyzed using the GeneSpring GX 10 software (Agilent Technologies). In a first step, the Agilent Feature Extraction software output was filtered on the basis of feature saturation, non-uniformity, pixel population consistency and signal strength relative to back ground level (Agilent Feature Extraction Manual). Only positively marked entities were accepted for further evaluation. All data was quantile normalized. In a second step, several quality control steps (e.g. correlation plots and correlation coefficients, quality metric plots and PCA) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality.

Differentially expressed genes from the microarray were determined using a Benjamini-Hochberg multiple correction-ANOVA test ($p<0.05$), followed by a TukeyHSD post-hoc test. The genes were considered differentially expressed when $p<0.05$ and the fold change (FC) ≥2. To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneGo, San Diego, CA, USA was used. Only those categories where $p<0.05$ were considered differentially altered. MetaCore TM (GeneGo, San Diego, CA, USA) was used to identify and visualize the involvement of the differentially expressed genes in specific pathways (FDR< 0.05). The microarray data used in this analysis have been submitted to NCBI GEO database under accession number GSE23156 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fnotryskkqyaoba&acc=GSE23156).
Data from microarray analysis, qRT-PCR, locomotor activity, comet assay and ROS assay were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov-Smirnov test and the variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p<0.05$) to compare treatment means with respective controls. If the data was not normally distributed, differences between treatments were assessed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. Results are given as mean ± standard error of mean. Differences were considered significant at $p\leq0.05$.

4.3 Results

4.3.1 PKC412 concentrations and embryo survival

PKC412 concentrations in the exposure water were measured at 0 h and 24 h in order to determine the actual exposure concentrations. The PKC412 concentrations measured independently in the different experiments are given in Table 2. In the microarray experiment,

actual concentrations were lower than nominal, and they decreased during exposure. Geometric mean concentrations (n=3) were 1.3 µg/L and 21 µg/L. Also in the behavioral experiment using the Multispecies Freshwater Biomonitor® the PKC412 concentration decreased during the 24 h exposure and the mean measured concentration was 2.1 µg/L and 31 µg/L. In the experiments, where the comet assay, TUNEL assay and ROS assay were performed, the initial concentrations were close to nominal, and decreased again during the 24 h exposure. The mean measured concentrations were 1.6 µg/L and 31 µg/L. No significant mortality was observed in any of the different experiments and treatment groups.

Table 2

Nominal and measured concentrations of PKC412 in exposure waters after 6 dpf of exposure for the microarray, Multispecies Freshwater Biomonitor® experiment and comet assay

Nominal Concentration	**Exposure waters**				
	Measured concentration (µg/L)				
	0h	**% of nominal**	**24h**	**% of nominal**	**Geometric Mean**
Microarray					
2 µg/L	1.50 ± 0.1	75.2	1.06 ± 0.2	52.9	**1.3**
40 µg/L	25.12 ± 1.6	62.8	17.20 ± 3.1	43.0	**21**
Multispecies Freshwater Biomonitor®					
2 µg/L	2.36 ± 0.1	117.8	1.90 ± 0.2	95.1	**2.1**
40 µg/L	37.45 ± 1.7	93.6	25.10 ± 1.7	62.7	**31**
TUNEL assay, comet assay, ROS assay					
2 µg/L	2.19 ± 0.2	109.5	1.23 ± 0.1	61.7	**1.6**
40 µg/L	38.60 ± 6.4	96.5	24.93 ± 5.2	62.3	**31**

4.3.2 Alteration of gene expression

Gene expression profiles derived from microarray results of control and PKC412-exposed embryos (n = 4) are based on 80 pooled individuals. As listed in Appendix 2 Table 1, 259 and 511 genes were differentially expressed in zebrafish eleuthero-embryos ($log_2 \geq 2$, $p<0.05$) after exposure to 1.3 µg/L and 21 µg/L PKC412, respectively. At 1.3 µg/L PKC412, 112 (43 %) genes were down-regulated, and 147 (57 %) up-regulated.

Of the 511 genes differentially expressed at 21 µg/L PKC412 130 (25%) genes were down-regulated, and 381 (75 %) were up-regulated. Only 101 of the significantly altered genes were regulated at both concentrations, however, all of them were regulated in the same direction. At both concentrations, most functional groups of genes (fold change (FC, $\log_2$) at least 2-fold, $p \leq 0.05$) were similar. Gene Ontology (GO) analysis was performed to identify functional groups of genes. GO defines terms representing gene product properties, and covers three domains namely cellular components, molecular functions and biological processes. At both concentrations, functionally identified genes fell into more than 1500 different categories. In Appendix 2 Table 2 the top 100 GO processes are listed. GO-categories including all kind of different detections or responses to stimuli are of particular importance e.g. detection of light stimulus, detection of abiotic stimulus, detection / response to external stimulus, visual perception etc. (Appendix 2 Table 2).

Additionally to the GO analysis, we performed a pathway analysis with MetaCore. The different treatments had 28 maps (1.3 µg/L PKC412: 29 maps; 21 µg/L PKC412: 34 maps) with their corresponding pathways in common (Appendix 2 Table 3). These maps include pathways for lipid biosynthesis and regulation, cholesterol and bile acid homeostasis, angiogenesis, vitamin and cofactor metabolism and its regulation, mitogenic signaling and apoptosis. For validation of these results, additional experiments were performed focusing on five significantly altered maps (Appendix 2 Table 3). Effects on angiogenesis were analyzed by performing an additional experiment with transgenic zebrafish. In addition, effects on apoptosis were analyzed by performing a TUNEL assay, DNA damage was assessed by a comet assay and oxidative stress response by a ROS assay.

In order to confirm the microarray results, qRT-PCR was performed for eight selected genes (Table 3, Figure 1). These genes belong to different pathways and were selected on their importance in different pathways: *abcc4* (ATP-binding cassette, sub-family C), *cry5* (cryptochrome 5), *ndrg1l* (N-myc downstream regulated gene 1, like), *opn1mw2* (opsin 1 (cone pigments), medium-wave-sensitive, 2), *pdpk1* 1 (3-phosphoinositide dependent protein kinase-), *per1* (period homolog 1), *sos* (similar to son of sevenless homolog 2), and *xpc* (xeroderma pigmentosum, complementation group C). A down-regulation occurred for *abcc4*, *cry5*, *per1*, and *xpc*, whereas an up-regulation was found for *ndrg1l*, *opn1mw2*, *pdpk* and *sos* (Figure 1). In all cases, except *sos*, the same tendency for up- or down-regulation occurred for the microarray and for qRT-PCR analysis.

Table 3

Fold changes of selected genes differentially regulated in zebrafish eleuthero-embryos determined by microarray and qRT-PCR after exposure to 1.3 µg/L and 21 µg/L PKC412. Values are expressed as average fold change. Asterisks show statistically significant difference to control ($p < 0.05$).

Gene name	Fold Change (log_2)				
	1.3 µg/L PKC412			21 µg/L PKC412	
	Array	qPCR		Array	qPCR
ATP-binding cassette, sub-family C **(abcc4)**	-2.00*	-1.21*		-2.98*	-1.03*
Cryptochrome 5 **(cry5)**	-2.68*	-2.35*		-2.86*	-2.12*
N-myc downstream regulated gene 1, like **(ndrg1l)**	4.00*	1.99*		4.10*	2.41*
Opsin 1 (cone pigments), medium-wave-sensitive, 2 **(opn1mw2)**	2.60*	0.45*		2.36*	1.00*
3-Phosphoinositide dependent protein kinase-1 **(pdpk1)**	2.60*	0.88*		1.71*	1.05*
Period homolog 1 **(per1)**	-9.80*	-3.58*		-6.41*	-3.16*
Similar to son of sevenless homolog 2 **(sos)**	0.85*	-0.27		2.03*	0.62
Xeroderma pigmentosum, complementation group C **(xpc)**	-2.90*	-1.40*		-2.20*	-0.55

4.3.3 Effects on the circadian rhythm and locomotor activity

As shown in Table 3, PKC412 altered the expression of genes regulating circadian rhythm e.g. *cry1-5* (1.3 µg/L PKC412: -2.08, 3.9, -4.4, 2.3, -2.7-fold change log_2, respectively; 21 µg/L PKC: -1.54, 2.7, -3, 1.8 -2.9-fold change log_2, respectively), *per1,2,4* (1.3 µg/L PKC412: -9.8, -2.04, -4.5-fold change log_2, respectively; 21 µg/L PKC: -6.4, -3.08, -3.17-fold change log_2, respectively)*, arntl* (1.3 µg/L PKC412: 2.5-fold change log_2; 21 µg/L PKC: 3.12-fold change log_2), *clock* (1.3 µg/L PKC412: 2.9-fold change log_2; 21 µg/L PKC: 2.7-fold change log_2) etc. In mammals, the circadian rhythm controls a diversity of behavioral and physiological rhythmic processes such as regulation of sleep wakefulness, secretion of hormones and locomotor activity (Hastings, 1997). In zebrafish, diazepam alters the same set of genes and influences the swimming behavior (Oggier et al., 2010).

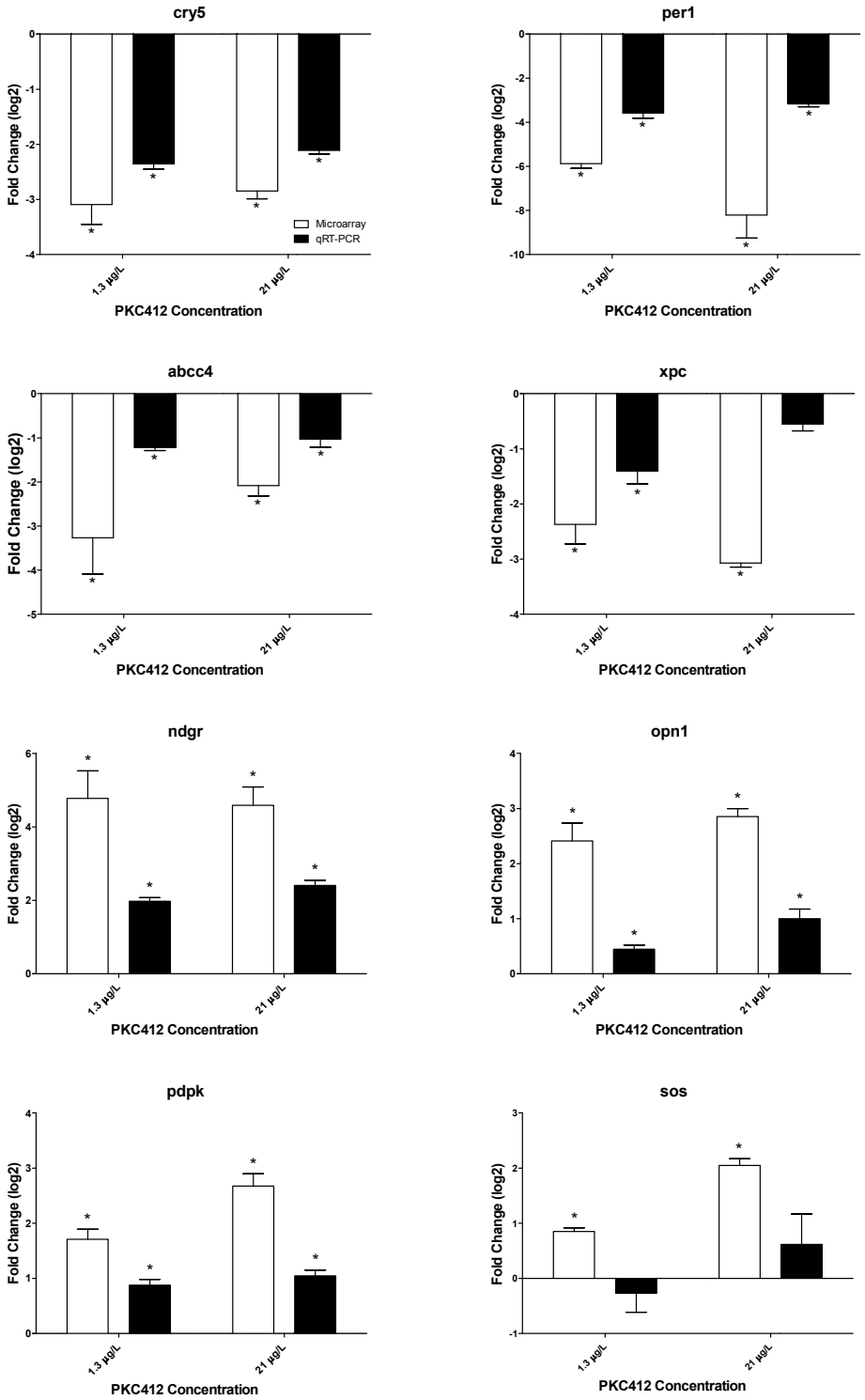

Figure 1

Comparison of gene expression in zebrafish larvae determined by microarray (n = 4 replicates, 80 eleuthero-embryos pooled, white bars) and qRT-PCR (n = 6, 80 eleuthero-embryos, black bars) after exposure to 1.3 µg/L and 21 µg/L PKC412, respectively. Values are expressed as average fold change (log_2) with standard error compared to control animals as indicated for selected genes. Asterisks (*) indicate statistically significant difference to control ($p < 0.05$).

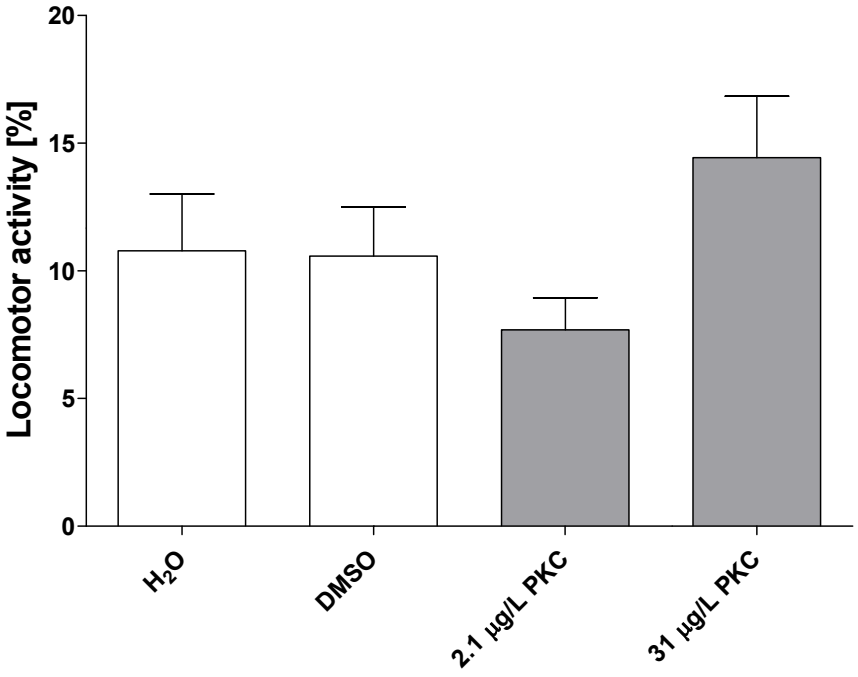

Figure 2

Comparison of the locomotor activity (percent of total time spent in locomotion during 2 h) of D. rerio eleuthero-embryos exposed for 6 dpf to 2.1 and 31 µg/L PKC412 or water control and solvent control. Asterisks (*) indicate significant differences between treatments ($p < 0.05$).

Therefore, an additional set of experiments was performed to evaluate whether changes found in the gene expression pattern of circadian genes in eleuthero-embryos were paralleled by changes in the locomotor activity. The behavioral experiments revealed that exposure of the embryos to PKC412 did not significantly affect their locomotor activity (Figure 2).

4.3.4 Effects on angiogenesis

Angiogenesis is an important process during cancerogenesis, which is affected by PKC412 in humans (Fabbro et al., 2000). We hypothesized that similar effects occur in zebrafish embryos during development. Important genes in angiogenesis are the vascular endothelial growth factor (*vegf*), which is down-regulated by PKC412 in humans (Fabbro et al., 2000), and angiopoietin *(ang)*. In our study we did not find a significant alteration in *vegf* transcript levels in zebrafish eleuthero-embryo (data not shown). However, there is a significant up-regulation of *ang2* (3.6-fold change $\log_2$) in the higher PKC412 concentration of 31 µg/L (Appendix 2 Table 1). *Ang2* is an antagonist of *ang1* and disrupts blood vessel formation when over-expressed (Maisonpierre et al., 1997). In addition to alterations in gene

expression, we also found a significant alteration of pathways involved in vascular development (Appendix 2 Table 3).

In order to link these findings with physiological changes in blood vessel development, we exposed (Tg(fli1a:EGFP)y1;Tg(gata1:dsRed)sd2) double transgenic zebrafish embryos to 2 µg/L and 40 µg/L PKC412. While focusing mostly on the trunk vasculature, we did not observe any abnormalities in cardiovascular development compared to control embryos. At 24 hpf, the dorsal aorta and the posterior cardinal vein developed normally and normal sprouting of the intersegmental vessels was observed. At 6 dpf all the main vessels looked virtually identical in treated and control embryos (Figure 3). Dorsal aorta, posterior cardinal vein and intersegmental vessels were normally developed and normal heart beat and blood flow was observed.

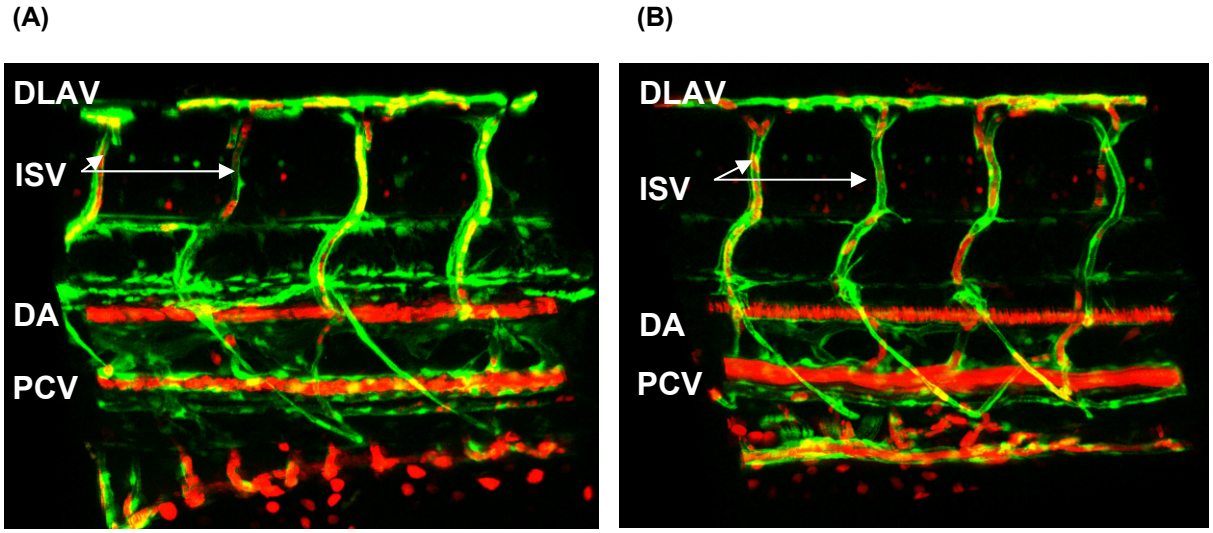

Figure 3

Vascular development is not affected by PKC412 treatment. The vascular system was visualized in a double transgenic zebrafish eleuthero-embryo (TG:fli1a:EGFPy1; gata1:DsRedsd2) on a Leica TCS SP5 Confocal Microscope. Endothelial cells are labelled in green and erythrocytes are labelled in red. ***(A)*** *DMSO solvent control and* ***(B)*** *40 µg/L PKC412. The pictures are maximal intensity projections of confocal z-stacks. DA – dorsal aorta, PCV – posterior cardinal vein, ISV – intersomitic vessel, DLAV – dorsal longitudinal anastomotic vessel. No major defects were detected in treated PKC412 embryos.*

25 double transgenic embryos from each treatment were analyzed in detail. We also looked at general morphology of the embryos. Single embryos from treated and control groups showed mild developmental abnormalities, which can occasionally be observed in non-treated fish, especially in transgenic lines. 5 of 25 embryos treated with 40 ug/L PKC412 had a slightly misshaped trunk with the tail bend dorsally. However, the vasculature of these embryos was normal (Figure 3). More analyses on wild type embryos would be needed to find out whether this misshaping is due to the drug treatment.

Therefore, PKC412 did not negatively interfere up to 6 days with the vascular development of zebrafish embryos at both concentrations. However, the vascular network at this stage is already very complicated and subtle differences might have been gone unnoticed.

4.3.5 Effects on apoptosis in olfactory placodes

PKC inhibitors can induce apoptosis in humans (Tenzer et al., 2001). Thereby the PI3K3/Akt pathway is of high importance as the cytotoxic effect of PKC412 is mediated by this pathway. In the apoptosis pathway the phosphoinositide dependent kinase PDK1 (also called PDPK1) leads to phosphorylation of v-akt murine thymoma viral oncogene homolog Akt, and therefore to its activation. Constitutively active Akt results in an enhanced protection against apoptotic cellular insults. In contrast to findings in humans, we found an up-regulation of *pdpk* (Appendix 2 Table 1, 2.6-fold change $\log_2$) in the lower PKC412 concentration.

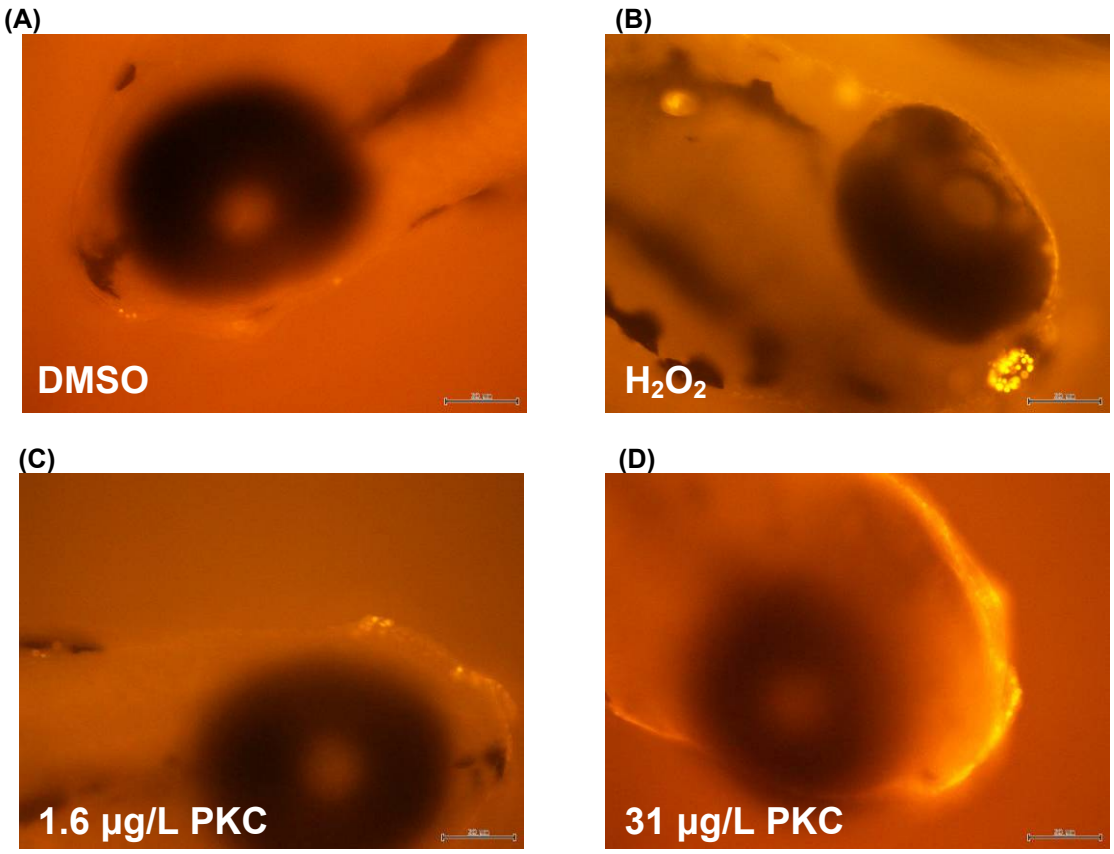

Figure 4

Zebrafish larvae exposed to 1.6 µg/L and 31 µg/L PKC412 until 144 hpf were stained with TMred. Apoptotic cells appeared mainly in the olfactory placodes. ***(A)*** *Solvent control,* ***(B)*** *positive control,* ***(C)*** *1.6 µg/L PKC412,* ***(D)*** *31 µg/L PKC412*

In addition, we found a down-regulation of transcripts of *xpc*, a gene that is important for DNA damage recognition. Additionally, apoptotic pathways are of significance (Appendix 2

Table 3). Based on these findings, we conducted a TUNEL assay to search for regions and tissue in the eleuthero-embryo, where apoptosis is induced by PKC412. Figure 4 illustrates that apoptosis mostly occured in the head region. The olfactory placode, which is important for the perception of odorants, is mainly affected. This finding is in parallel to the observed up-regulation of gene transcripts of some odorant receptors *or102-3* (2.8-fold change log_2) and *or11-10* (2.2-fold change log_2) found by microarray analysis for the highest PKC412 concentration (Appendix 2 Table 1), suggesting a repair of these affected cells.

4.3.6 Effects on DNA damage

PKC412 led to significant down-regulation of *xpc*. At 1.6 µg/L and 31 µg/L PKC412 we found a 2.9- and 2.2-fold change log_2, respectively, in *xpc* transcripts (Appendix 2 Table 1, Figure1). Additionally, there was a down-regulation of *apex* nuclease 1 (-2.7-fold change log_2) at 31 µg/L PKC412. As *xpc* is important in DNA damage recognition and APEX1 for DNA repair, we conducted a comet assay to evaluate whether this alteration occurs not only on the transcription, but also on the physiological level. Further support for potential DNA damage induced by PKC412 comes from the fact that pathways involved in DNA damage response were significantly affected (Appendix 2 Table 3). The comet assay demonstrates a dose-dependent increase in DNA damage in eleuthero-embryos exposed to PKC412 (Figure 5). Significant DNA damage occurred in the embryos at 31 µg/L PKC412 (Figure 5). At the lower concentration of 1.6 µg/L PKC412 a significant increase in tail length, but not in % DNA in tail, tail moment and olive tail moment occurred (Figure 5).

4.3.7 Effects on formation of reactive oxygen species (ROS)

MetaCore pathway analysis indicated that there is an alteration in oxidative stress regulation. To evaluate whether this is related to increased formation of ROS by PKC412, we performed an additional experiment to determine formation of ROS. The results show that the ROS-levels remained unchanged at both PKC412 concentrations as compared to the control (Figure 6) and thus PKC412 is not inducing ROS.

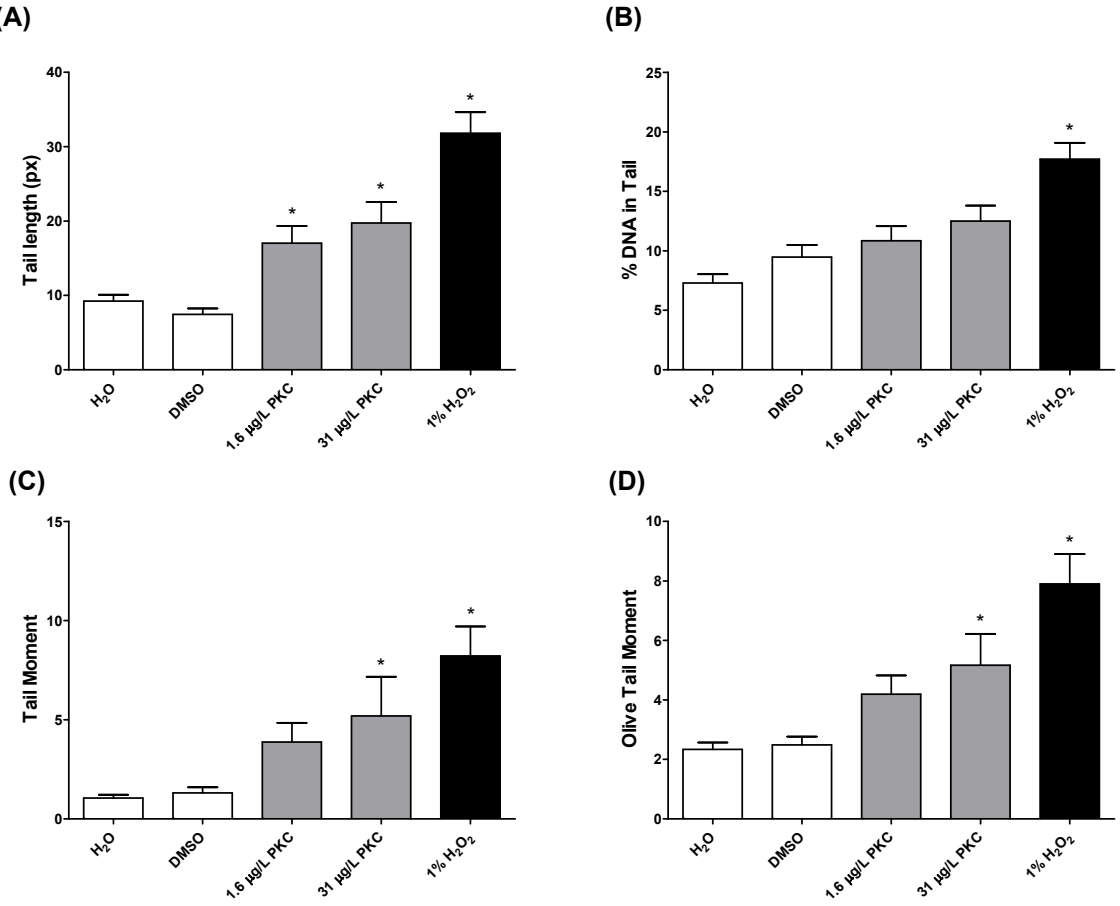

Figure 5

Comet assay in zebrafish eleuthero-embryos exposed to 1.6 µg/L and 31 µg/L PKC412. ***(A)*** *Tail length,* ***(B)*** *% DNA in Tail,* ***(C)*** *Tail Moment and* ***(D)*** *Olive Tail Moment were calculated using Comet score. 4 slides per dosage were examined and 24 cells per slides were scored. The values are presented as the mean ± SEM. Asterisks (*) indicate values that are significantly different from the control (Kruskall-Wallis test followed by posthoc Dunn's multiple comparison test; $p < 0.05$).*

4.4 Discussion

This is the first study investigating the effects of PKC412 on gene expression on non-target organisms in aquatic systems. We show effects of PKC412 at low concentrations in zebrafish eleuthero-embryos. Thereby we demonstrate that human pharmaceuticals exhibit their effects in aquatic organisms by similar molecular modes of action as in humans due to target conservation in vertebrates. We also tested the hypothesis that alteration in gene expression propagate into physiological effects.

PKC412 was designed as tyrosine kinase inhibitor with a broad inhibition spectrum of the human kinome. It was shown that at high-affinity interactions (Kd < 100 nM), this compound was not highly selective for kinases (Karaman et al., 2008). Consequently, it is not surprising that PKC412 alters more than 500 genes in zebrafish eleuthero-embryos at a concentration

of 21 µg/L. Furthermore, the broad spectrum of kinases inhibited by the non-selective PKC412 makes the interpretation of the results challenging due to the involvement of many cellular pathways.

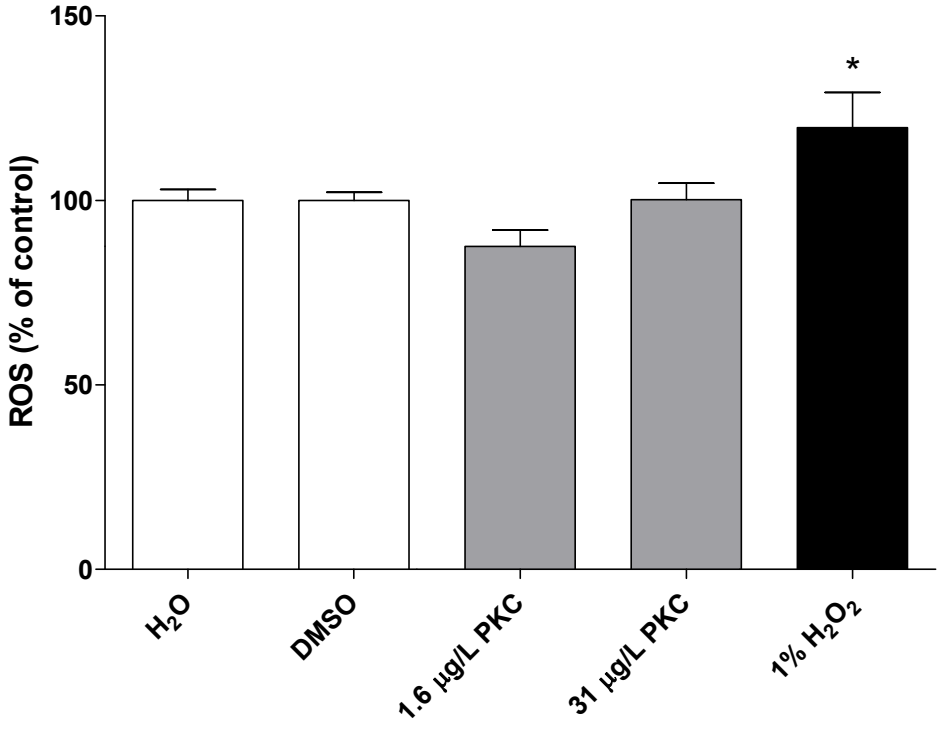

Figure 6

Effects of 1.6 µg/L and 31 µg/L PKC412 on ROS formation after 144 hpf of exposure. The values are presented as the mean ± SEM. Asterisks (*) indicate values that are significantly different from the control (ANOVA followed by a posthoc test: Tukeys's multiple comparison test; $p < 0.05$)

In humans PKC412 inhibits FLT3, PDGF receptors, VEGF and c-kit by preventing the tyrosine autophosphorylation (Fabbro et al., 2000). In addition, this compound inhibits P-glycoprotein mediated multidrug resistance in tumor cells. When comparing our results with human effects and gene expression changes obtained in MV4-11 cells (human lymphoblast cells) after treatment with 570 µg/L PKC412 (Stolzel et al., 2010), some similarities show up. In zebrafish eleuthero-embryos there were also alterations of solute carriers as in human cells (Stolzel et al., 2010), albeit a solute carrier of family 2 was significantly down-regulated in the lower concentration only. In addition, we similarly found a significant down-regulation of the ABC-transporter *abcc4*, which is important for the efflux of anti-cancer drugs. However, there was no alteration of *flt3, pdgfr, vegf* and *c-kit* at the transcript level in zebrafish embryos. Binding of PKC412 leads to inhibition of these kinases, therefore we hypothesize that PKC412 does not interfere with gene expression but protein function. One important identified GO-category is visual perception. Expression of genes encoding proteins involved in vision such as chrystallins and opsins were significantly altered by PKC412. Crystallins are

important for the protection against lens opacity (Clark, 2004), and Opsins are important for dim-/daylight-vision and color-vision (Takechi and Kawamura, 2005). These findings in zebrafish are similar to results obtained in mice and humans after treatment with PKC412. There are several VEGF and PDGF receptors in the retina. The inhibition of these receptors can lead to decreased epiretinal membrane formation in mice (Saishin et al., 2003) and reduction in macular edema in humans (Campochiaro, 2004). Therefore, up-regulation of Chrystallins and Opsins may be a response to the inhibition of VEGF and PDGF receptors.

In addition to visual perception, we also found that responses to light and external/internal stimuli are important targets of PKC412. As the circadian rhythm is dependent on stimuli, namely Zeitgeber, there is a significant alteration of those genes at both PKC412 concentrations in zebrafish. It is known that in mice several protein kinases C are expressed in the hypothalamic suprachiastic nuclei (SCN) (Van der Zee and Bult, 1995), the region which is important for circadian processes. Therefore, protein kinases C play an important role in the mammalian circadian rhythm, and inhibition of PKC can lead to phase advances (Schak and Harrington, 1999; Jakubcakova et al., 2007). As PKC is already expressed in the early zebrafish development (Slatter et al., 2005; Patten et al., 2007), it could be affected in the SCN. Additionally, it was shown in the marine dinoflagellate *Lingulodinium polyedrum* that staurosporine (precursor of PKC412) leads to phase-shifting and increases the free running period of the circadian rhythm (Comolli and Hastings, 1999). At the behavioral level altered expression of clock genes is correlated with changes in the locomotor activity (Jakubcakova et al., 2007). For this reason we investigated the effects of PKC412 on the locomotor activity. However, PKC412 had no significant effect on the locomotor activity in our experimental set-up. A reason could be that an observation over 2 h is too short to obtain significant effects. Also the time-point when the measurements took place could have an influence. We performed our measurements at day-light, and effects would have probably been more pronounced at night.

In addition to exploring effects on different GO-categories we performed a pathway analysis in order to elucidate altered maps and pathways after PKC412 exposure. Out of the 28 maps we chose four different maps with their corresponding pathways to perform additional experiments. It is known from humans that PKC412 can have effects on angiogenesis and apoptosis including DNA damage repair. However, there are no indications from mammalian studies that PKC412 exposure can lead to the formation of reactive oxygen species.

The need for new blood vessels is important during embryogenesis, as well as in processes during cancerogenesis (Carmeliet and Jain, 2000). The formation of blood vessels and

evaluation of blood flow can be observed in zebrafish eleuthero-embryos, rendering it an ideal model organism to study angiogenesis. As angiogenesis is affected by PKC412 in humans (Fabbro et al., 2000), we hypothesize that similar effects occur in zebrafish eleuthero-embryos. Thereby, angiopoietins play an important role (Pham et al., 2001; Kubota et al., 2005). In fact, we found a significant up-regulation of *ang2*. It was shown that over-expression of *ang2* can lead to blood vessel disruption in the mouse embryo (Maisonpierre et al., 1997). There is also a simultaneous regulation of VEGF and angiopoietin. However, angiopoietins do not participate in the initial vascular phase of vascular development, but rather play an important role in angiogenic outgrowth, vessel remodeling and maturation (Dumont et al., 1994; Suri et al., 1996). Our results obtained after the exposure of double transgenic zebrafish eleuthero-embryos support the findings that angiopoietins do not participate in the initial vascular phase. In addition, our findings are in agreement with those obtained after exposure of zebrafish eleuthero-embryos to 57 µg/L for 24 hpf (Chan et al., 2002). PKC412 did not generate measurable antiangiogenic effects during the exposure time. It may be possible that there are strain differences between zebrafish that account for our failure to detect vascular defects in transgenic fish. However, our results are in line with the microarray data in the sense that we did not find any alteration in *vegf* transcript levels. In addition, the defects in the vasculature might be too subtle to detect upon PKC412 treatment, so that the went unnoticed in our assays.

PKC inhibitors can induce apoptosis in humans (Tenzer et al., 2001). To protect mammals from apoptotic stress stimuli, the phosphatidylinositol 3'-kinase (PI3K)/Akt survival pathway is important. In this pathway Akt needs to be phosphorylated by the phosphoinositide dependent kinase PDK1, which was found to be down-regulated in mice after treatment with staurosporine (Hill et al., 2001). In mice PKC412 can mediate its cytotoxic effects partly via down-regulation of this pathway. Additionally, PKC412 decreased the site-specific phosphorylation of Akt, required for its activity (Tenzer et al., 2001). In contrast to these findings, we found an up-regulation of the upstream *pdpk1* at the lower PKC412 concentration. This leads to the hypothesis that there is no down-regulation of the Akt pathway but a protection against apoptosis. However, there is the indication that the failure in DNA damage recognition (down-regulation of *xpc*) is more important, and therefore apoptosis is induced in limited regions of the eleuthero-embryo. The TUNEL assay showed that apoptotic cells were located in the olfactory placodes (Figure 4). As there is also an up-regulation of odorant receptors, which are mainly expresssed in the olfactory placodes, we suggest that this is a reaction to the damage and associated to repairing the loss of cells. Yet, there is no evidence for this phenomenon in mammals.

DNA damage can be paralleled with apoptosis. To evaluate whether alterations in *xpc* and *apex* can be linked to DNA damage, we performed comet assays. There is a clear link between gene expression alteration and DNA damage at both PKC412 concentrations. Although there is not significantly more DNA in the tail, there is a significant increase in tail length indicating that DNA damage took place. Data from the comet assay demonstrate that gene expression changes in DNA damage recognition are paralleled with intracellular DNA damage. This data is in agreement with the results obtained in human lymphoblast cells after treatment with PKC412 (Seedhouse et al., 2006).

We also showed that physiological outcomes of maps of low significance, as the regulation of oxidative stress, do not correlate with the obtained gene expression. Nevertheless, several markers such as metallothionein (*mt*, 2.3-fold down-regulated at 21 µg/L) and glutathione S-transferase M (*gstm*, 1.1- and 1.2-fold down-regulated at 1.3 and 21 µg/L, respectively) were significantly altered. It is known that mt is up-regulated by free radicals in rainbow trout cells (Kling and Olsson, 2000). Our findings on the alteration of *gst* are in contrast with those obtained in goldfish after treatment with gemfibrozil, which is supposed to down-regulate the antioxidant defense system (Mimeault et al., 2006). However, the gene expression of other markers such as the superoxide dismutase (*sod*) or the catalase (*cat*) was not altered.

In our study we demonstrate that the response of zebrafish eleuthero-embryo to different PKC412 concentrations is partly similar to the response in humans. Significant changes in gene expression occurred at low concentrations of 1.3 µg/L PKC412. This demonstrates that alterations in gene expression are more sensitive than mortality and physiological effects. Additionally, the gene expression profile points to the modes of action of PKC412 in zebrafish. Due to the very broad kinase inhibition spectrum of PKC412 a correlation to physiological alterations is difficult. In some cases gene expression changes were not paralleled by physiological changes at low concentrations and sometimes even at high concentrations. This raises the question whether and when gene expression changes propagate to toxicological relevant measures such as reduction of the ability to find food, to reproduce or to escape from predators.

In conclusion, the present study confirms that the toxicogenomic approach provides important data to identify and characterize molecular effects and to investigate potential modes of action of an antineoplastic agent. This allows for establishing new biomarkers, not regularly assessed in routine ecotoxicological studies. PKC412-induced alterations of gene transcripts were partly paralleled by physiological effects at high, but not at low PKC412 concentrations that may be of environmental relevance. Our study also shows that molecular

studies should be paralleled with ecotoxicological investigations of known ecological relevance to interrelate sensitive changes in gene expression to physiological effects.

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We declare that Birgit Hoeger is employed by Novartis Pharma AG, Basel, Switzerland who owns the patent on PKC412. However, the authors have no conflicts of interest.

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Chapter 5

Global gene expression profile induced by the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) in zebrafish (*Danio rerio*)

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Abstract

Residues of the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) are ubiquitously found in aquatic biota, but potential adverse effects in fish are fairly unknown. To identify molecular effects and modes of action of EHMC we applied gene expression profiling in zebrafish using whole genome microarrays. Transcriptome analysis and validation of target genes were performed after 14 days of exposure of male zebrafish. Concentrations of 2.2 µg/L and 890 µg/L EHMC lead to alteration of 1096 and 1137 transcripts, respectively, belonging to many pathways. Genes involved in lipid metabolism and estrogenic pathway (*vtg1*), lipid biosynthesis (*ptgds*), vitamin A metabolic process (*rbp2a*), DNA damage and apoptosis (*gadd45b*), and regulation of cell growth (*igfbp1a*) were investigated by qRT-PCR analysis in whole body, liver, brain and testis. The analysis showed tissue-specific gene profiles and revealed that EHMC slightly affects the transcription of genes involved in hormonal pathways including *vtg1*, *esr1*, *esr2b*, *ar*, *cyp19b* and *hsd17β3*.

Key words: UV-filter, EHMC, *Danio rerio*, microarray, transcriptional changes

5.1 Introduction

Personal care products are increasingly used in daily life and chemical residues enter aquatic systems, where they may lead to adverse effects in biota. UV-absorbing chemicals (UV-filters) are widely used in sunscreens and in a wide variety of cosmetics (lipsticks, shampoos, creams, fragrances, skin lotions, hair sprays). In addition, UV-protection is also applied in numerous materials and products, where UV-filters (Balmer et al., 2005; Fent et al., 2010a,b) or benzotriazole UV-stabilizers (Nakata et al., 2009) are used. UV-filters are organic (e.g. 2-ethyl-hexyl-4-trimethoxycinnamate, EHMC) or inorganic (TiO2, ZnO) ingredients of personal care products, whose purpose is to filter UV-A and/or UV-B radiation from sunlight in order to protect the human skin and products, respectively, from negative effects.

Currently, 28 UV-filters are registered in the European Union (Zenker et al., 2008; Schlumpf et al., 2008). As a result of their various applications, UV-filters enter the aquatic environment either directly via wash-off from skin and clothes, or via effluents of wastewater treatment plants or swimming pool waters, where rather high levels of different UV-filters were measured (Balmer et al., 2005; Zwiener et al., 2007; Rodil and Moeder, 2008). Further sources of UV-filter residues are landfill leachates, sewage sludge, as well as deposition from building parts, which are protected with coatings (Plagellat et al., 2006).

Many organic UV-filters are lipophilic, photostable and relatively stable in the aquatic environment. They adsorb into sewage sludge (Plagellat et al., 2006), and some of them bioaccumulate in aquatic biota. 2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC), benzophenone-3 and 4-methoxycinnamate were demonstrated to accumulate in fish (Buser et al., 2006; Fent et al., 2010a), and in human breast milk (Schlumpf et al., 2008). The acute toxicity of some UV-filter increases with the log Pow (lipophilicity) of the compound in *D. magna* showing an LC_{50} value of 0.28 mg/L for EHMC (Fent et al., 2010b). Since significant amounts of these chemicals are used today, there are health concerns, and therefore, more knowledge is needed for a better understanding of potential toxicological effects and on the modes of action of these compounds.

Some UV-filters interfere with the sex hormone system and may act as endocrine disruptors. Hormonal activity was documented *in vitro* (Schlumpf et al., 2001; Kunz and Fent, 2006a) as well as *in vivo* (Kunz et al., 2006a). In fathead minnows, the UV-filters 3-benzylidene camphor and benzophenone-2 exhibit estrogenic activity and adverse effects on fertility and reproduction (Kunz et al., 2006a,b; Weisbrod et al., 2007). Benzophenone-3 (BP-3) led to induction of vitellogenin in rainbow trout and Japanese medaka (Coronado et al., 2008), and

benzophenone-4 (BP-4) was demonstrated to interfere with the sex hormone system displaying multiple hormonal activities in zebrafish (*Danio rerio*) (Zucchi et al., 2010).

EHMC is one of the most widely used UV-filters and included in more that 90% of commercial topically applied sunscreens formulation (Diaz-Cruz et al., 2008). The environmental concentration of EHMC ranges between 0.01-0.1 µg/L in treated wastewater, and up to 19 µg/L in untreated municipal wastewater (Balmer et al., 2005). EHMC residues were detected in lakes and rivers (Balmer et al., 2005; Fent et al., 2010a) and in coastal seawater up to 390 ng/L were reported (Langford and Thomas, 2008). Recently up to 3 µg/L EHMC were found in a lake (Rodil et al., 2009), and 0.26 to 5.61 µg/L were reported in drinking water (Loraine and Pettigrove, 2006). Unexpectedly, even at very remote environments such as the Pacific Ocean (Polynesia) this compound was detected (Goksoyr et al., 2009). EHMC is lipophilic (logP = 5.66; Zenker et al., 2008) and accumulates in aquatic biota (Fent et al., 2010a). Residues were found in different trophic levels showing a tendency for bioaccumulation along the food-chain, with concentrations up to 340 ng/g lipids in cormorants (Fent et al., 2010a).

Despite its widespread presence in the environment, little is known about the potential risk posed by EHMC to aquatic life. In recombinant yeast systems EHMC showed anti-estrogenic and anti-androgenic activities, combined with weak androgenic activity (Kunz and Fent, 2006b). Injection of high concentrations of EHMC in male medaka led to induction of vitellogenin (VTG) (Inui et al., 2003), while no significant VTG induction was observed in juvenile fathead minnows exposed to lower aqueous concentrations (Kunz and Fent, 2006b). Despite these studies the potential endocrine activity and molecular modes of action of EHMC in fish remain elusive and need further investigation. In light of the importance and increasing use of this UV-filter, and considering that potential hormonal activities and adverse effects on fertility are of concern, there is a need for a better understanding of potential environmental risks associated with EHMC contamination.

Since changes in gene expression often precede cellular, physiological and toxicological responses, analysis of gene expression profiles upon exposure may be a sensitive tool for investigating adverse effects of pollutants including their molecular mode of action (Oggier et al. 2010, 2011). Thus far, toxicogenomics was applied mainly with compounds whose modes of action are known (e.g. Lettieri, 2006; Hoffmann et al., 2006, 2008). However, microarray analysis focusing on thousands of genes also offers the potential to analyse for the molecular effects of chemicals with unknown modes of action. By employing global gene expression analysis, in the present study, we focus on EHMC to elucidate its unknown mode of action in

order to shed a new light on its potential effects in fish. Whole-adult microarray analysis performed on a small vertebrate such as zebrafish might represent a strategy to potentially obtain a large amount of *in vivo* data on the transcriptome of the entire organism (Lam et al., 2008), and thus to obtain insight into the mode of action of EHMC.

The aim of our present study is to evaluate the overall molecular effects of EHMC by analysing the whole body of adult male zebrafish. This allows an overall fingerprint of EHMC as a sum effect in all tissues, which is in contrast to analysis of only one tissue alone, hard to select without knowledge about the target tissues of the compound. Data from previous studies suggest that EHMC interferes with the sex hormone system (Inui et al., 2003; Kunz and Fent 2006b). Additionally, by analyzing transcriptional changes of selected target genes belonging to different pathways including vitellogenin 1 (*vtg1*), prostaglandin D2 synthase (*ptgds*), insulin growth factor binding protein 1a (*igfbp1a*), retinol-binding protein type II (*rbp2a*), and growth arrest and DNA-damage-inducible beta (*gadd45b*), we aimed at comparing expressional changes in the whole body to those in the liver, brain and testis. To analyse for potential hormonal effects, we focused on expressional changes of more classical genes involved in hormonal pathways and steroidogenesis including vitellogenin 3 (*vtg3*), ERalpha (*esr1*), ERbeta1 (*esr2b*), androgen receptor (*ar*), hydroxysteroid 17-ß dehydrogenase-3 (*hsd17ß3*), P450aromB (*cyp19b*) and P450aromA (*cyp19a*).

By following an approach to analyse for unknown molecular effects of EHMC by means of global expression analysis (microarrays), and by following a targeted gene expression approach (qRT-PCR) focusing on mainly hormonal pathways in multiple tissues, we demonstrate the involvement of several pathways in response to EHMC exposure.

5.2 Materials and Methods

5.2.1 Chemicals

2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC, CAS No. 5466-77-3) and 3-(4-methylbenzylidene)-camphor (4MBC; CAS No. 36861-47-9) were purchased from Merck (Glattbrugg, Switzerland), ethanol (EtOH), methanol and dichloromethane in HPLC grade from J.T. Baker (Stehelin AG, Basel, Switzerland). Formic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Fluka AG, Buchs, Switzerland). All compounds were >99% pure. The water solubility of EHMC is 0.2 mg/L at 20 ˚C (http://www.merck-chemicals.com), and the log P is 5.66 (Zenker et al., 2008). Master concentrated stock solution of 30 and 0.3 g/L EHMC were made up in DMSO and stored in the dark at 4°C

between uses. After dilution with tank water (10L), DMSO concentrations did not exceed 0.01% in each tanks used in the experiment.

5.2.2 Analytical chemistry

During the experiment aliquots of exposure water were taken three times randomly from the tanks to determine the actual exposure concentration of EHMC. Water samples of each replicate were taken at the beginning (0 h), after 24 h and prior to water renewal (48 h). This was done on days 1 to 3, 7 to 9 and 11 to 13, respectively, from different replicate tanks. The water samples were stored in the dark at -20 °C until analysis by HPLC-DAD. Extraction of water samples and chemical analysis was performed according to Kunz et al. (2006b).

5.2.3 Maintenance of adult zebrafish

Adult zebrafish (*Danio rerio*) (>121 days) were obtained from a local dealer (Swisstropicalfish, Niederönz, Switzerland), transferred to culture tanks (300 L) and acclimatized one month in our laboratory prior to commencing the experiments. Fish of both sexes were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 µS/cm. The water temperature was held constant at 27±1 °C with the photoperiod set at 16:8 h light/dark. Fish were fed twice daily with TetraMin flakes (Tetra GmbH, Melle, Germany) and once a day with a combination of brine shrimp (*Artemia salina*) and white mosquito larvae.

5.2.4 Exposure of adult male zebrafish

Adult male zebrafish were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated water (12 fish/ tank). The experimental setup consisted of 4 groups: 3 µg EHMC/L, 3000 µg EHMC/L, solvent control (SC, 1 ml of DMSO in 10 litres of reconstituted water) and water control (reconstituted water). Each dose-group and the control consisted of four replicates. The quality of the exposure water was continuously monitored by measuring the oxygen concentration (>70%), pH value (6.7-7.2) and temperature (27±1 °C). During the experiment, appearance, mortality and abnormal behaviour of fish were recorded daily.

Fish were exposed for 14 days to EHMC in a semi-static renewal procedure; every 48 h fish were transferred to new tanks containing the appropriate EHMC concentrations. During the exposure period fish were fed daily as previously described. At the end of the experiment, fish were euthanized in a clove oil solution (Fluka AG, Buchs, Switzerland), the total body

length and weight were measured. For whole-body microarray analyses and subsequent quantitative real-time polymerase chain reaction (qRT-PCR) confirmation, two fish per replicate of every group were snap-frozen in liquid nitrogen after sex determination.
Zebrafish sex determination was carried out opening the belly to ensure testis presence before collecting fish for further molecular analysis.

A number of 10 fish of each replicate of each dose group were used for qRT-PCR analysis, and liver, brain and testis were immediately excised. Pools of tissues of ten fish per replicate were collected, placed in RNA*later* and stored at −80 °C for subsequent total RNA extraction.

5.2.5 RNA isolation, array hybridization and sample selection

For microarray analysis one whole fish per replicate of each dose group was pounded in a ceramic mortar to powder with liquid nitrogen, and subsequently equally transferred into two Eppendorf tubes for subsequent total RNA extraction using RNeasy Mini Kit (Qiagen, Basel, Switzerland).

For qRT-PCR analysis of tissues, total RNA was extracted from pools of 10 fish of liver, brain and testis using RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 UV-VIS spectrophotometer at 260 nm. The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing a 260/280 nm ratio between 1.8- 2.1, a 28S/18S ratio between 1.5- 2 and an RNA integrity number (RIN) > 8 were processed further.

For transcriptom analysis, a total of 16 arrays (Agilent 4 × 44 K Zebrafish microarray) were used, including four for the 3 µg/L, four for the 3000 µg/L EHMC dose group, four for the solvent control group (DMSO) and four for the water control group. Total RNA samples (600 ng) were reverse-transcribed into double-strand cDNA in the presence of RNA poly-A controls with the Agilent One-Color RNA Spike-In Kit. Cy3 labeling and hybridization were performed according to the manufacturer's manual.

After reverse-transcription of RNA into double-stranded cDNA, double-strand cDNA was *in vitro* transcribed into cRNA in the presence of Cy3 labeled nucleotides using a Low RNA Input Linear Amp Kit +Cy dye (Agilent Technologies, Basel, Switzerland), performed at the Functional Genomic Centre (ETHZ and University of Zürich, Switzerland). The Cy3-labeled cRNA was purified using an RNeasy mini kit (Qiagen, Basel, Switzerland), and quality and quantity was determined using a NanoDrop ND-1000 UV-VIS Spectrophotometer and an

Agilent 2100 Bioanalyzer, respectively. Only cRNA samples with a total cRNA yield higher than 2 µg and a dye incorporation rate between 9 pmol/µg and 20 pmol/µg were used for hybridization. Cy-3-labeled cRNA samples (1.65 µg) were mixed with Agilent blocking solution, subsequently fragmented randomly to 100-200 bp at 65 °C with fragmentation buffer and resuspended in hybridization buffer as provided by the gene expression hybridization Kit (Agilent Technologies). Target cRNA samples (100 µL) were hybridized to the Agilent Zebrafish 4 x 44K Gene Expression Microarray for 17 h at 65 °C. The hybridized arrays were then washed using Agilent GE wash buffers 1 and 2 according to the manufacturer's instructions and scanned by an Agilent Microarray Scanner (Agilent p/n G2565BA) at 5 µm resolution with the green photomultiplier tube set to 100% and a scan area of 61 x 21.6 mm. Image generation and feature extraction was performed using the Agilent Feature Extraction (FE) software version 9.5.3. Quality control was additionally considered before performing the statistical analysis. These included array hybridization pattern inspection: absence of scratches, bubbles, areas of non-hybridization, proper grid alignment, spike performance in controls with a linear dynamic range of 5 orders of magnitude and the number of green-feature non-uniformity outliers which should be below 100 for all samples.

5.2.6 qRT-PCR analysis

Changes in the expression of selected genes identified as being different following microarray analysis were validated using real-time reverse transcription-polymerase chain reaction (qRT-PCR). The selected genes included, vitellogenin 1 (*vtg1*), insulin-like growth factor binding protein 1(*igfbp1*), prostaglandin D2 synthase (*ptgds*), growth arrest and DNA-damage-inducible beta (*gadd45b*) and cellular retinol-binding protein type II (*rbp2a*) (Table 1). In addition, seven target transcripts were chosen for qRT-PCR analysis, because of their involvement in hormonal pathways and steroidogenesis. They included vitellogenin 3 (*vtg3*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), hydroxysteroid 17-ß dehydrogenase-3 (*hsd17ß3*), androgen receptor (*ar*), P450aromB (*cyp19b*) and P450aromA (*cyp19a*).

Gene-specific primers of all the genes were obtained either from published zebrafish primers sequences, or designed based on zebrafish sequences available at NCBI (http://www.ncbi.nlm.nih.gov/) (Table 1). Primer sequences not previously published were designed using IDTDNA software (www.idtdna.com).

Amplicons were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide, in order to confirm correct product size and primers specificity. Total RNA from individual whole body fish and pools of 10, each of zebrafish liver, brain and testis were isolated as described above (*n* = 4).

Table 1

Primer sequences for quantitative real-time PCR analysis and sources

Target gene	GenBank number	Sense primer (5'-3')	Antisense primer (5'-3')	Product size (bp)
Vtg1 [a]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94
Vtg3 [b]	AF254638	TTAGAACCAGCAAAGGATGC	CATCTCTTTTCTCCTTAAATAC	208
esr1 [c]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163
esr2b [d]	NM_174862.3	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT	80
ar [e]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237
hsd17ß3 [f]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121
cyp19b [g]	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94
cyp19a [g]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92
gadd45b	NM_213031	GGGACGAACATTTTGAAGGA	AACACGGTCCTTTTCAGTGC	131
rbp2a	AF363957	GGAGATGCTCAGCAATGACA	TCTGCACAATGACCTTCGTC	110
ptgds	NM_213634	CCATCAAGACCAAAGGAGGA	TCCATTTTGTGGAAGCATGA	152
Igfbp1 [a]	NM_173283	GTCATCCTGGAATGGGAAGA	TGTGTGACGGATCAGTGGTT	93
RpL13α [h]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100

vtg1 (vitellogenin 1), ***vtg3*** (vitellogenin 3), ***esr1*** (ERalpha), ***esr2b*** (ERbeta1), ***ar*** (androgen receptor), ***hsd17ß3*** (hydroxysteroid 17-ß dehydrogenase-3), ***cyp19b*** (P450aromB), ***cyp19a*** (P450aromA), ***gadd45b*** (growth arrest and DNA-damage-inducible beta), ***rbp2a*** (retinol-binding protein type II), ***ptgds*** (prostaglandin D2 synthase), ***igfbp1*** (insulin growth factor binding protein 1) and ***RpL13α***. **a** Hoffmann, et al., 2006 **b** Meng et al., 2010 **c** Martyniuk et al., 2007 **d** Chandrasekar et al., 2010 **e** Hossain et al., 2008 **f** Hoffmann, et al., 2008 **g** Arukwe et al., 2008 **h** Oggier et al., 2010

RNA samples used for qRT-PCR analysis were further treated with RNase free DNase set (Qiagen, Basel, Switzerland) to purify the RNA preparations from DNA contamination and to subsequently remove DNase and divalent cations from the samples.

Subsequently, 1 µg of total RNA template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and desoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. The cDNA was used to perform SYBR-PCR based on SYBR-

Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland).

The real-time PCR program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 57–60 °C, depending on transcript target as shown in Table 1 (30 s) and 72 °C (30 s), followed by a melting curve analysis post run. All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland).

For determining the efficiencies of the PCR reactions, reaction mixtures with template diluted 1:10 in five steps were also run and the slopes of the regression curves were calculated. For calculating expression levels of selected genes, mRNA normalization was performed against the housekeeping gene (*RpL13α*). The ΔC_T values were calibrated against the control ΔC_T values for both target genes. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), all gene expression data are reported as $\log_2$-transformed.

5.2.7 Data analysis and statistics

To identify lists of differentially-expressed genes (DEGs) in this study, the text outputs (raw microarray data) obtained from Functional Genomic Centre Zurich (FGCZ) were imported into GeneSpring GX 11 (Agilent Technologies). In a first step, the Agilent Feature Extraction software output was filtered on the basis of feature saturation, non-uniformity, pixel population consistency and signal strength relative to back ground level (Agilent Feature Extraction Manual). Only positively marked entities, in which at least 50% of the values for any out of the three conditions were accepted for further evaluation. All data were quantile normalized. In a second step, several quality control steps (e.g. correlation plots and correlation coefficients, quality metric plots and PCA) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality.

Differentially expressed genes from the microarray were determined using a Benjamini-Hochberg multiple correction-ANOVA test ($p<0.05$), followed by a TukeyHSD post-hoc test. The genes were considered differentially expressed when $p<0.05$ and the fold change (FC) was ≥2.

To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneGo (GeneGo, San Diego, CA, Version 6.3, http://www.genego.com) was used. Enrichment was examined in all three major GO categories (e.g., biological process,

cellular component, molecular function), but only biological process results are reported here, as they were the most relevant category for the purposes of this study. Only those categories where $p<0.05$ are considered differentially altered.

MetaCoreTM (GeneGo, San Diego, CA, Version 6.3) from GeneGo Inc. http://www.genego.com was used to identify and to visualize the involvement of the differentially expressed genes in specific pathways (FDR< 0.05).

Data from qRT-PCR were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov-Smirnov test and the variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p<0.05$) to compare treatment means with respective controls. Results are given as mean ± standard error of mean. Differences were considered significant at $p\leq0.05$.

5.3 Results

5.3.1 EHMC exposure concentrations and gross behaviour

Concentrations of EHMC in experimental water were determined by HPLC-DAD after 0, 24, and 48 h of exposure for each treatment group. At the nominal EHMC concentration of 3 µg/L, measured concentrations were 2.2 µg/L at 0 h, 3.6 µg/L at 24 h, and not detectable (n.d.) at 48 h. At the nominal EHMC concentration of 3000 µg/L measured concentrations (median±SD) were 1741±23 µg/L (n=3) at 0 h, and decreased to 890±9 µg/L (n=3) at 24 h, and to 140±2 µg/L (n=3) at 48 h. This gives a median of the actual exposure concentration over this time period of 2.2 and 890 µg/L (Table 2). The data demonstrate that actual concentrations of EHMC (expressed as median concentrations) were lower than nominal and further decreased during exposure.

During the 14-d experiment, no mortality occurred even in the highest dose group and there were no indications of abnormal behaviour during the exposure. No significant alteration of condition factors (CF) was noted (Figure 1).

Table 2

Nominal and median of actual concentrations of EHMC in exposure waters at different time points (0, 24 and 48 hour).

Nominal Concentration (µg/L)	Exposure waters			
	Measured concentration (µg/L)			
	0 h	24 h	48 h	Median**
0 (n=3)	0*	0*	0*	**0**
3 (n=1)[1]	2.2	3.6	0	**2.2**
3000 (n=3)	1741± 23*	890 ±9*	140±2*	**890**

* median±standard deviation of replicates

** median of actual exposure concentration after 0, 24 and 48 h

[1] only one replicate could be measured due lack of sampling water evaluated

5.3.2 Differential expression of genes in whole body determined by microarrays

All microarray hybridizations in this experiment met our quality requirements and were included in the analysis. For gene expression profiles determined by microarrays, four individual whole body samples, each of control, DMSO-solvent control and EHMC-exposed male fish, were analysed. Exposure to 2.2 µg/L and 890 µg/L EHMC resulted in a differential expression of 1096 and 1137 genes (fold change ≥2, $p<0.05$), respectively, as listed in Appendix 3 Table 1. At 2.2 µg/L EHMC, 594 (54.2 %) genes were down-regulated, and 502 (45.8 %) up-regulated. Of the 1137 genes differentially expressed at 890 µg/L EHMC 699 (61.48 %) were down-regulated, and 438 (38.52 %) up-regulated. A total of 708 of the significantly altered genes were regulated at both EHMC concentrations, and they were always regulated in the same direction (up or down) (Appendix 3 Table 1). The Venn Diagram (Figure 2) indicates that the expression of 388 and 429 genes were differently altered (fold change ≥2, $p<0.05$) by 2.2 and 890 µg/L EHMC, respectively.

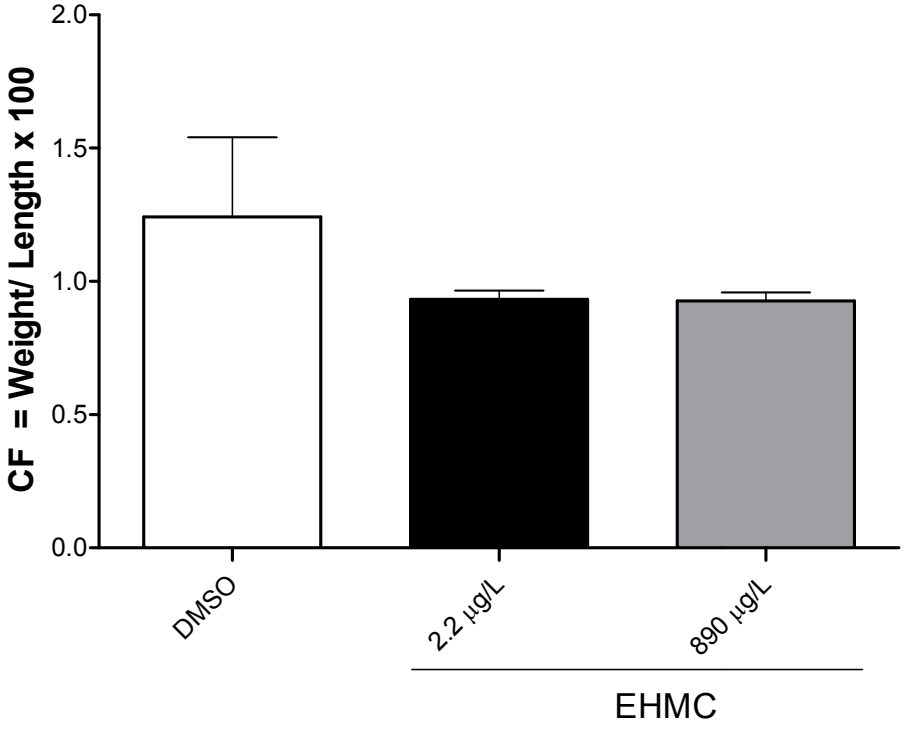

Figure 1

Condition factor (CF) of male zebrafish. Controls (white bar) and fish exposed to 3 µg/L and 3000 µg/L EHMC (black and grey bar). Values are means ± SEM (n = 12 males). Nominal median EHMC concentrations are given.

The Gene Ontology (GO) provides a controlled way to describe gene products in three categories, namely cellular components, molecular functions and biological processes (Ashburner et al., 2000). GO terms for biological processes were examined to determine the function of genes with altered patterns of expression. At both concentrations, functionally identified genes fell into 1650 different categories. 'Cellular Process' was the most significantly overrepresented Biological Process GO term (Appendix 3 Table 2). The top 50 GO processes are listed in Appendix 3 Table 2. GO-categories show that mainly cellular processes, development processes, system development, multicellular organismal development, anatomical structure development, muscle system process, muscle contraction and response to hormone stimulus are affected at both EHMC concentrations (Appendix 3 Table 2).

Additionally to the GO analysis, we performed a pathway analysis (GeneGo Pathway Maps) with MetaCore TM. The different treatments showed 33 maps (2.2 µg/L EHMC: 33 maps; 890 µg/L: 36 maps) with their corresponding pathways in common (Appendix 3 Table 3). The 890 µg/L EHMC treatment displayed 3 additional maps, namely, retinoid signalling, hypoxia response regulation and visual perception.

The 15 most relevant maps shown in Appendix 3 Table 3 include pathways involved in tissue remodeling and wound repair, immune system response, inflammatory response, cell differentiation, DNA-damage response, cell cycle and its regulation, apoptosis, blood clotting, protein synthesis, calcium signalling, vasoconstriction, vascular development (angiogenesis), mitogenic signalling, protein degradation and androgen signalling.

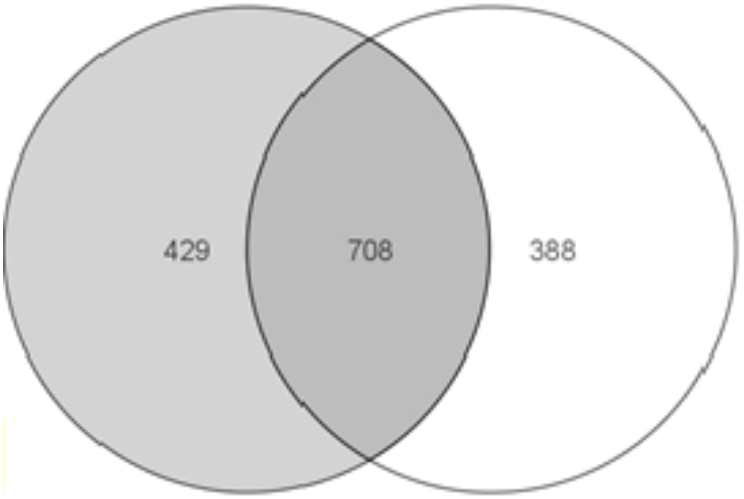

Figure 2

Venn diagram illustrating the number of altered genes that are in common between the two EHMC exposures (708 genes), and number of genes that are only regulated by 2.2 (388) and 890 (429) µg/L EHMC, respectively.

5.3.3 Validation of microarray data by real-time qRT-PCR assessment of target genes

To validate gene expression changes determined by microarrays, five genes involved in different pathways were selected. They include genes belonging to hormonal pathways such as vitellogenin 1 (*vtg1*), lipid metabolism such as the prostaglandin D2 synthase (*ptgds*), a gene that mediates cell growth, and known to be induced by estrogenic compounds (Riley et al., 2004; Hoffman et al., 2006; Martyniuk et al., 2007; Baker et al., 2009) such as the insulin growth factor binding protein 1a (*igfbp1a*), a gene involved in vitamin A metabolism, the retinol-binding protein type II (*rbp2a*), and a gene involved in apoptosis, DNA damage and immune response such as the growth arrest and DNA-damage-inducible beta (*gadd45b*). The latter was previously found to be modulated by EE2 (Hoffmann et al., 2006).

These genes, *ptgds*, *igfbp1a*, *rbp2a* and *gadd45b* (except for *vtg1*, MetaCore software visualize mouse, rat, worm, fly, yeast and dog data on networks) were then used to build corresponding networks using MetaCore, as illustrated in Figure 3 A, B, C and D.

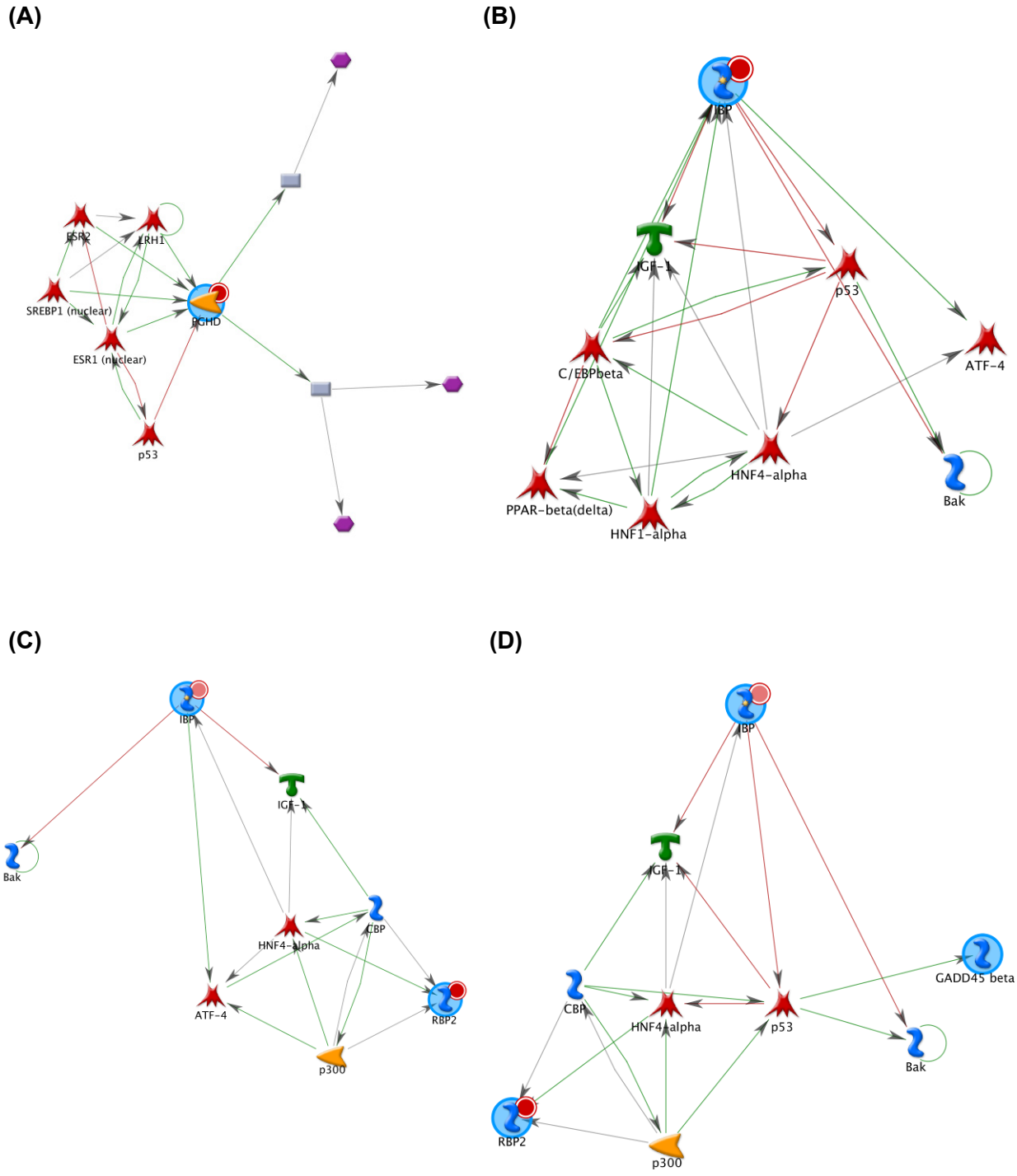

Figure 3

A, B, C, D: Gene networks for ptgds (A), igfbp1a (B), rbp2a (C) and gadd45b (D) as constructed by MetaCore version 6.4.

These selected genes were validated by qRT-PCR in whole-body samples. In addition, these five genes were analysed in different tissues including liver, brain and testis to compare their expression in individual organs with those in the whole body (Table 3).

The observed mRNA alterations occurred always in the same direction in the whole body in both the microarrays and qRT-PCR measurements. This demonstrates that the transcriptional changes determined by microarrays are paralleled by qRT-PCR measurements, but the magnitude of the fold increase of these transcripts was more pronounced in the microarray analysis.

Different trends in expressional changes were observed in whole-body samples, and different tissues (Table 3). The *vtg1* mRNA was significantly down-regulated in the whole body at both EHMC concentrations, and this was also noted in the brain and testis. Conversely, a dose-related *vtg1* up-regulation occurred in the liver (Table 3). It is important to highlight that for the *vtg1* transcript the microarray data demonstrate a down regulation for 3 *vtg1* partial sequences (as for example agilent probe ID: A_15_P110740; Appendix 3 Table 1). Based on BLAST (http://blast.ncbi.nlm.nih.gov/) comparisons and ClustalW multiple sequence alignment (http://www.ebi.ac.uk/Tools/clustalw/) we confirmed that the 3 partial *vtg1* sequences correspond to the *Danio rerio* gene *vtg1* with accession number NM_001044897.

Similar to the *vtg1* transcript, the *ptgds* transcript showed a different expression in the liver as compared to the whole body and the other tissues. This transcript was up-regulated in the whole body, brain and testis at both concentrations, while in the liver, it was down-regulated (Table 3). In the brain we observed a tendency for induction, whereas in the testis a significant up-regulation occurred, especially at 890 µg/L EHMC.

A dose-related and parallel increase occurred for the *igfbp1a* transcript in the whole body, and in the liver, brain and testis (Table 3). In brain and testis a considerable up-regulation is noted at 890 µg/L EHMC. The *rbp2* mRNA was down-regulated in the whole body and in all of the investigated tissues. The microarray data show that *rbp2* mRNA was strongly down-regulated in the whole body at 2.2 µg/L EHMC (Table 3). A significant induction was noted at both EHMC concentrations for the *gadd45b* transcript in the whole body, whereas the alteration was not significant in individual tissues (Table 3).

Table 3

Fold changes (Log_2) of selected genes differentially regulated in zebrafish adult male determined by microarray (GeneSpring normalization) and qRT-PCR ($2^{-\Delta\Delta Ct}$ normalization) after exposure to 2.2 µg/L and 890 µg/L EHMC. Values are expressed as average fold change. Asterisks show statistically significant difference to control ($p < 0.05$).

		2.2 µg/L EHMC					890 µg/L EHMC				
		Whole fish	Whole fish	Liver	Brain	Testis	Whole fish	Whole fish	Liver	Brain	Testis
GenBank	Description	Array	qRT-PCR	qRT-PCR	qRT-PCR	qRT-PCR	Array	qRT-PCR	qRT-PCR	qRT-PCR	qRT-PCR
NM_001044897	vitellogenin 1 (***vtg1***)	-5.03*	-1.66*	1.05*	-0.60*	-0.57*	-2.96*	-3.31*	1.39*	-0.63*	-0.69*
NM_213634	prostaglandin D2 synthase (***ptgds***)	1.75*	1.35*	-0.90	0.51	1.58*	2.54*	1.93*	-0.57	0.70	2.38*
NM_173283	insulin-like growth factor binding protein 1a (***igfbp1a***)	1.46*	0.71	1.03*	0.83*	1.66*	1.18*	1.82*	1.48*	3.24*	3.62*
AF363957	retinol-binding protein type II (***rbp2a***)	-5.08*	-0.51	-0.60	-2.53*	-0.23	-3.69*	-0.97*	-0.55	-1.60*	-0.59
NM_213031	growth arrest and DNA-damage-inducible, beta (***gadd45b***)	2.80*	2.26*	-1.17	-0.74	-0.83	1.61*	0.98*	-0.42	0.61	-0.42

5.3.4 Transcriptional changes of selected target genes in tissues

Based on our previous study (Zucchi et al., 2010) seven additional candidate genes were chosen for the evaluation of molecular effects of EHMC with a focus on hormonal activity and steroidogenesis to test the hypothesis that this UV-filter acts on these processes. The selected genes included vitellogenin 3 (*vtg3*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), androgen receptor (ar) and hydroxysteroid 17-ß dehydrogenase-3 (*hsd17ß3*). Their expression levels were evaluated by qRT-PCR in the whole body, liver, brain and testis (Figure 4; Figure 5 A, B, C; Figure 6). Additionally, differential expressions of P450aromB (*cyp19b*) and P450aromA (*cyp19a*) were determined in brain and testis, respectively. The selected genes belong to three categories including an estrogen-responsive gene (*vtg3*), nuclear receptors (*esr1*, *esr2b*, *ar*) and steroid metabolism (*hsd17ß3*, *cyp19b* and *cyp19a*).

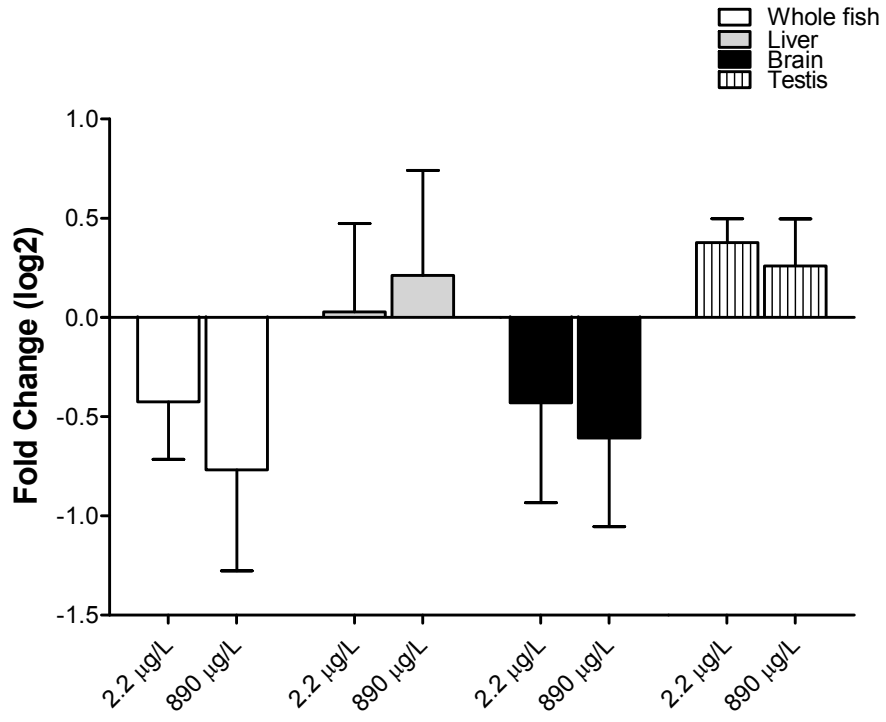

Figure 4

Relative gene expression of vtg3 in whole body, liver, brain and testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in vtg3 abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ *method. Results are given as the mean value ± standard deviation (n=4 replicates for adults).*

The modulation of *esr1* and *esr2b* transcripts in whole-body and tissue samples show an almost identical trend and similar expression pattern. An induction occurred in the whole body and liver, whereas the transcripts were down-regulated in brain and testis (Figure 5A and B). The alterations were mostly significant at 890 µg/L EHMC, except for the brain, were the down-regulation was significant at 2.2 µg/L. The *vtg3* transcript was not significantly altered in all the investigated tissues (Figure 4).

The *ar* transcript was significantly down-regulated in the whole body at 890 µg/L (Figure 5C). A dose-related down-regulation was also noted in the liver (Figure 5C), whereas in the brain, a slight but significant up-regulation occurred at 890 µg/L EHMC. The *hsd17ß3* transcript was significantly down-regulated in whole-body samples in a dose-dependent manner (Figure 6).

In the brain the transcription of *cyp19b*, encoding the aromatase B, was significantly induced at 890 µg/L EHMC (Figure 7). However, the amount of *cyp19a* transcripts, encoding aromatase A, did not significantly change after EHMC treatment (Figure 8).

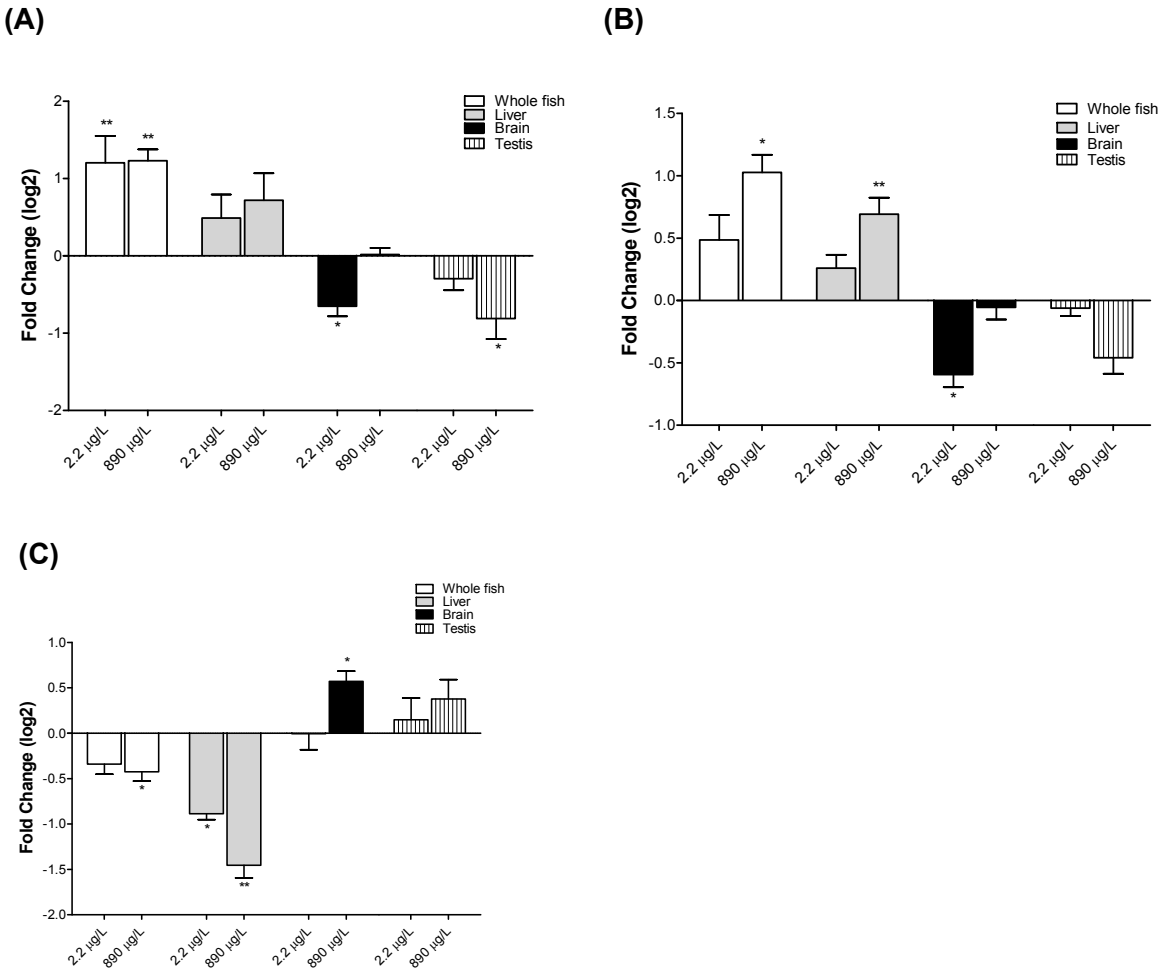

Figure 5

*Relative gene expression of (A) esr1, (B) esr2b and (C) ar in whole body, liver, brain and testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in esr1 abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (n=4 replicates for adults). Asterisks indicate significantly higher expression than control (*p< 0.05), and (**p<0.01).*

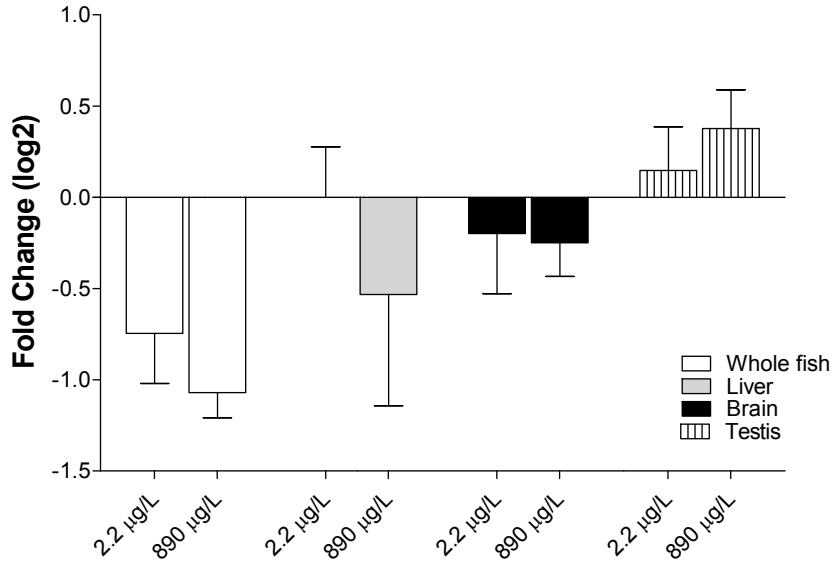

Figure 6

*Relative gene expression of hsd17ß3 in whole body, liver, brain and testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in hsd17ß3 abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (n=4 replicates for adults). Asterisks indicate significantly higher expression than control (*p< 0.05), and (**p<0.01).*

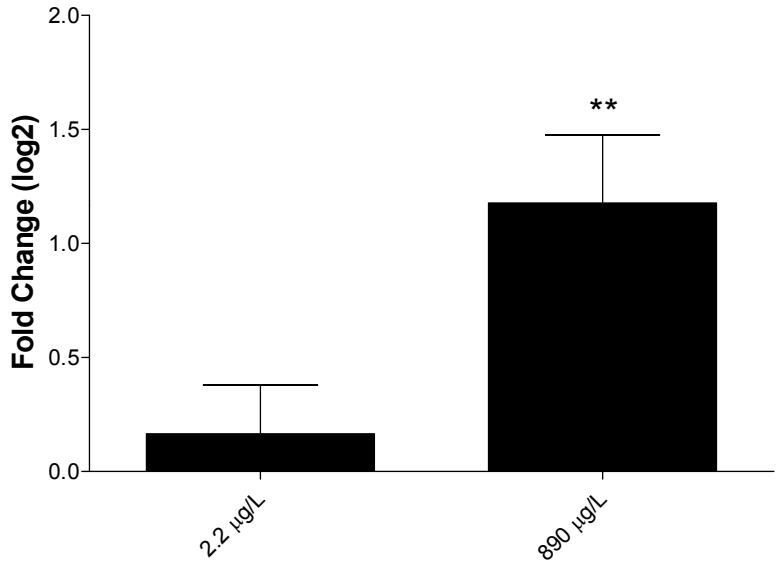

Figure 7

*Relative gene expression of cyp19b in brain of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in cyp19b abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (n=4 replicates for adults). Asterisks indicate significantly higher expression than control (*p< 0.05), and (**p<0.01).*

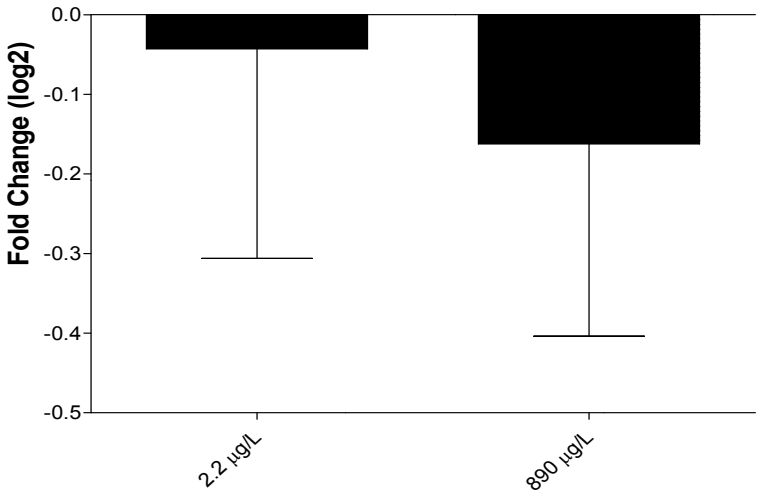

Figure 8

Relative gene expression of cyp19a in testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in cyp19a abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (n=4 replicates for adults).

5.4 Discussion

In the present study we examined molecular effects of EHMC in zebrafish by applying gene expression profiling and a targeted gene expression approach. Global gene expression profiling was applied in the whole body and alterations of selected microarray-detected target genes were validated by means of qRT-PCR. Therefore, we determined the expression of a suite of target genes to compare the response in the whole body to that in individual tissues. Based on previous *in vitro* and *in vivo* studies (Kunz and Fent 2006b; Inui et al., 2003) indicating a hormonal activity, we additionally chose target genes to test the hypothesis that EHMC interferes with genes involved in sex hormonal signalling and steroidogenesis.

We analyzed the response in whole organisms, because this captures the total sum of the transcriptional changes in the entire organism, and thus the overall molecular effect giving indications on the mode of action of EHMC on the transcriptome. However, at the same time, this approach has its limitations, such as loss of weak signals or signals from small tissues, and the tissue-specific contributions cannot be unravelled. The microarray data demonstrate a high number of expressional changes in the whole-body samples and associated biological pathways. As in the case of our present study, they do not necessarily provide a defined, clear and simple picture of affected processes, making data interpretation challenging. Despite these limitations, we have chosen this promising approach as the predictive power of

whole-organism genomics has been demonstrated in zebrafish exposed to polycyclic aromatic hydrocarbons and estrogenic compounds (Lam et al., 2008). This approach also provides a fingerprint of all expressional changes within the organism, as the specific target organs of EHMC are unknown. This is in contrast to our previous studies on pharmaceuticals having a known mode of action (Oggier et al. 2010, 2011). In addition, we chose a target gene approach to scrutinize potential mode of actions, with emphasis on hormonal pathways.

5.4.1 Microarray analysis and gene categorization in whole organism

Exposure to EHMC altered the expression of a considerable number of genes in the entire organism. A comparison of the transcriptional profiles in whole bodies and different tissues demonstrated that the overall expression pattern and tissue-specific profiles do not necessarily match.

A total of 1096 and 1137 genes were altered at 2.2 µg/L and 890 µg/L EHMC, respectively, which is in the same range of genes affected in the liver of zebrafish after exposure to 17α-ethynylestradiol (EE2) (Hoffmann et al., 2006). Complex interrelationships in expressional changes with most genes showing differential responses between tissues (and sexes) were also reported upon exposure of fathead minnows to 17ß-estradiol (E2) (Filby et al., 2006).
EHMC influences mainly the biological process (GO:008150) and molecular function (GO:003674) as highlighted by GO terms classification. At both EHMC concentrations differentially altered genes belong to many biological processes indicating a diverse effects pattern. The top 50 GO processes include cell processes (e.g.: cell death, cellular metabolic process, cell cycle and transmembrane transport), developmental process (e.g.: multicellular organismal development, anatomical structure development), muscle system process and contraction (e.g.: muscle cell differentiation, smooth muscle contraction), response to hormone stimulus (e.g.: response to steroid hormone stimulus, response to chemical stimulus), response to endogenous stimulus (e.g.: response to epidermal growth factor stimulus, response to hormone stimulus), regulation of multicellular organismal process (e.g.: regulation of angiogenesis, regulation of nervous system development, regulation of cytokine production), tissue development (e.g.: multicellular organismal development, organ development), among others (Appendix 3 Table 2). According to the GO processes, the major altered pathways by EHMC are mainly involved in tissue remodeling, wound repair, immune system response, inflammatory response, cell differentiation, DNA-damage response, cell cycle and its regulations and apoptosis.

Often GO analysis can be useful in providing information about the modes of action of a chemical, but the many GO terms affected by EHMC exposure are general descriptions,

which based on the results of this experiment alone without phenotypic anchoring, makes it difficult to link them to a specific mode of action. The differentially expressed genes involve many pathways indicating that EHMC rather than affecting just a few key pathways exhibits distributed effects across several biological processes and pathways. This is in contrast to our previous study on the neuropharmaceutical diazepam, which selectively affects few processes related to its known mode of action in zebrafish brain (Oggier et al., 2010).

We critically screened the genes involved in the most altered pathways by EHMC. Interestingly, we found genes such as talin 1 (*tln1*; NM_001009560) and transcripts of many homeobox genes to be affected by EHMC. In particular, *hoxa13b* (NM_131194), *hoxb6b* (NM_131538), *hoxa10b* (NM_131155) were increased at both EHMC concentrations, while the expression of *hoxb6a* (NM_131119) and *hoxc6b* (NM_131119) was reduced. EHMC lead to down-regulation of *tln1*; this gene encodes a cytoskeletal protein, which is concentrated in areas of cell-substratum and cell–cell contacts. The down-regulation by EHMC is in agreement with the observed affected tissue remodelling and wound repair pathways. In contrast, EE2 was found to induce *tln1* in stickleback (Katsiadaki et al., 2010), and estrogenic compounds are effective regulators of cell migration via cytoskeleton alteration (Acconcia et al., 2006; Giretti and Simoncini, 2008).

Hox genes play an important role in controlling development processes and patterning of the body axis during embryogenesis but hox genes are also expressed in the adult organism (Morgan, 2006). Expressional changes of hox genes suggest effects of EHMC on developmental processes, but functions and consequences of hox genes in the adult fish remain unclear. A similar pattern (although not involving all the same genes) occurred in the zebrafish telencephalon exposed to EE2 (Martyniuk et al., 2007). Moreover, alteration of hox gene expression in whole-body homogenates occurred in zebrafish after exposure to E2 (Cohen et al., 2008). Several hormones regulate hox gene expression (Daftary and Taylor, 2006), and estrogens including diethylstilbestrol (Akbas et al., 2004) and methoxychlor (Fei et al., 2005) alter hox expression. These data suggest that EHMC also acts similarly as hormonally active compounds on these genes. In addition, we found xeroderma pigmentosum, complementation group C, (*xpc*; NM_001045210), excision repair cross-complementing rodent repair (*ercc1*; NM_001103138), proliferating cell nuclear antigen (*pcna*; NM_131404), fanconi anemia complementation group L (*fancl*; NM_212982), replication protein A2 (*rpa2*; NM_131711), ezrin like (*ezrl*; NM_001020490), wingless-type MMTV integration site family, member 7Aa (*wnt7a*; NM_001025540), phosphatase and tensin homolog B (*ptenb*; NM_001001822), mitogen-activated protein kinase 1 (*map2k1*; NM_213419) and glutathione S-transferase pi (*gstp1*; BC083467) being altered in both

EHMC dose groups. The alteration of these genes, in particular those involved in nucleotide excision repair (NER) pathway, such as *xpc*, *ercc1*, *fancl*, *rpa2*, *pcna* and *gadd45b* are in agreement with the observed modulated pathways such as DNA-damage response, apoptosis and inflammatory response. This is in accordance to data in human cell lines, where *xpc* and *ercc1* involved in cellular response to DNA damage repair was altered after exposure to EHMC (Duale et al., 2010). NER is an important DNA repair system that eliminates a wide variety of helix-distorting DNA base lesions. EE2 led to a decrease in hepatic NER gene expression in adult male zebrafish including *xpc* (Notch et al., 2007), similarly to the effects displayed by EHMC in the present study.

5.4.2 Validation of microarray data

Based on the results obtained from GeneSpring normalization, canonical pathways analysis, and based on literature we choose 5 target-genes including *vtg1*, *ptgds*, *igfbp1a*, *rbp2a* and *gadd45b* to validate the microarray results by means of qRT-PCR. We found that the mRNA alterations occurred always in the same direction in the whole body, both in microarrays and qRT-PCR. This demonstrates the validity of the microarray data.

5.4.3 Differentially regulated genes in whole body, liver, brain and testes

The differential expression profile of the chosen genes upon exposure to EHMC is often tissue-specific. For some genes including *igfbp1a* and *rbp2a*, similar changes were observed in the whole body and different tissues, for the other chosen genes, however, a differential expression occurred (Table 3; Figure 5, Figure 6 A, B, C). As for the selected genes, the data obtained by microarrays seem more similar to the data obtained by qRT-PCR in the brain than those in the liver. To a lesser extent this also holds for the testis, where we observed the highest variability in terms of Ct value and the lowest responsiveness among the selected genes. The expressional changes were, however, not paralleled in the liver, which is often analysed (Sumpter and Jobling 1995; Hoffmann et al., 2006; Filby et al., 2007). This indicates that the molecular effects and modes of action of a compound cannot sufficiently be described in one organ alone, but rather needs a multi-organ approach for a more complete understanding. This has also been shown in fathead minnows (Pimephales promelas) experimentally exposed to E2 (Filby et al., 2006).

A similar responsiveness of some transcripts selected from microarrays was observed between whole body and the tissues. This holds for the expression of genes involved in regulation of growth and proliferation (*igfbp1a*), and those involved in vitamin A biosynthesis (*rbp2a*), which are also modulated by estrogens (Hoffmann et al., 2006; Martyniuk et al.,

2007). The alterations by EHMC (up-regulation of *igfbp1a*, down-regulation of *rbp2a*, Table 3) are similar in the whole-body and in all the investigated tissues (liver, brain and testis), although not to the same extent. A similar pattern, up-regulation of *igfbp1a* and down-regulation of *rbp2a*, was found after exposure of zebrafish to EE2 (Hoffmann et al., 2006), which suggests an estrogenic activity of EHMC towards these genes and processes.

A well established biomarker for estrogenic activity is the induction of *vtg1* and its protein, which is tissue-, stage- and sex-specific (Sumpter and Jobling 1995; Tyler and Sumpter, 1998). Whole-body microarray data in zebrafish show that among other genes *vtg1* expression occurs after exposure to estradiol, diethylstilbestrol and bisphenol A (Lam et al., 2008). In our study, we observed a down-regulation of *vtg1* transcripts in the whole body by EHMC. However, the differential expression profile was not equal in all the tissues. Similar to the whole body, *vtg1* expression was down-regulated in brain and testis, whereas a dose-related up-regulation occurred in the liver. Thus far, induction of *vtg1* by estrogens has mainly been investigated in the liver (Islinger et al., 2003; Wang et al., 2005; Hoffman et al., 2006; Sun et al., 2009; Levi et al., 2009). Consequently, our data suggest an estrogenic activity of EHMC in the liver, which is supported by the parallel induction of *esr2b*. On the other hand, down-regulation of *vtg1* in the whole body, brain and testis suggests an antiestrogenic activity of EHMC in these extrahepatic tissues. This finding is supported by recent findings in fish, where estradiol and BP-4 induced *vtg1* not only in liver, but also in heart and brain (Yin et al. 2009; Ma et al., 2009; Zucchi et al., 2010). An alteration of *vtg* expression cannot univocally be ascribed to an estrogenic response as its induction occurs by hypoxia (Wu, 2003), and recently Zhang et al. (2010) demonstrated that vitellogenin plays an important role in innate immune responses.

In the whole body, liver, brain and testis EHMC led to a dose-dependent up-regulation of the insulin-like growth factor binding protein 1a (*igfbp1a*). This transcript was selected in our study, because of its alteration by estrogens in fish (Riley et al., 2004; Hoffman et al., 2006; Martyniuk et al., 2007; Baker et al., 2009). Our data on EHMC are in agreement with previous studies in fish that showed alteration of *igfbp1a*, a transcript involved in cell growth. However, the *igfbp1a* up-regulation cannot univocally be ascribed as a pure estrogenic response, since other physiological factors are also inducers (Kelley et al., 2002; Riley et al., 2004). Further studies should evaluate, whether the alteration of genes involved in growth and proliferation will result in effects on these processes.

A gene involved in vitamin A biosynthesis, the retinol binding protein type II (*rbp2a*), was down-regulated by EHMC in the whole body and brain (and in liver and testis, although not

significantly). The expression of retinol binding protein (*rbp*) has been proposed as a possible biomarker for exposure to endocrine disrupting compounds (Levy et al., 2004). Similar to our observation with EHMC, the expression of *rbp2a* was down-regulated by EE2 in zebrafish (Hoffmann et al., 2006), and rainbow trout (Sammar et al., 2001). The effect of EHMC on *rbp2a* and thus vitamin A biosynthesis, metabolism and transport seems to represent an estrogenic response, but this needs to be further evaluated on a physiological level.

The prostaglandin D2 synthase catalyzes the conversion of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2) and the expression of *ptgds* is androgen-regulated (Love et al., 2009). This transcript is down-regulated after exposure to (EE2) (Hoffman et al., 2006), but also by the androgen 17ß-trenbolone (Dorst et al., 2009). In contrast, in our study EHMC led to up-regulation of *ptgds* in the whole body and testis. Similarly, an up-regulation was observed in flounder (*P. americanus*) experiencing multiple stress (Straub et al., 2004).

EHMC led to significant induction of DNA-damage-inducible beta (*gadd45b*) in the whole body, which is in agreement with the pathways identified including apoptosis and growth arrest. Similarly, EE2 led to a significant up-regulation of this transcript in zebrafish liver (Hoffmann et al., 2006). No significant differential expression of *gadd45b* occurred in the tissues analysed. Consequently, its transcription is induced in tissues other than liver, testis and brain.

5.4.3 Effects of EHMC on genes involved in hormonal pathways and steroidogenesis

EHMC led to a significant down-regulation of *vtg1*, which was not the case for *vtg3*, although the same trend was observed. Estrogens alter the expression of both *vtg1* and *vtg3* in fish (Miracle et al., 2006), although the responsiveness may differ (Islinger et al., 2003; Martyniuk et al., 2007).

The estrogen receptor genes *esr1* and *esr2b* were significantly up-regulated by EHMC in the whole body, which seems in contrast to the down-regulation of the *vtg1* transcript (as parallel induction occurs with estrogens). In contrast, down-regulation of *vtg1* was paralleled with *esr1* and *esr2b* down-regulation in the brain. In the liver, however, *esr2b* up-regulation would support the observed hepatic *vtg1* induction. The differential effect of EHMC on *vtg1* expression in different tissues highlights the importance for analyzing transcription profiles in multiple tissues.

In the whole body and liver, we observed a down-regulation of ar expression, while a slight up-regulation occurred at 890 µg/L EHMC in the brain. Filby et al. (2007) found no modulation of the ar transcript after EE2 exposure in male fathead minnows, while a strong down-regulation in hepatic ar mRNA was observed after exposure to the antiandrogen flutamide. This suggests an anti-androgenic activity of EHMC in the liver, and possibly in other organs.

EHMC-exposure slightly affected the expression of genes involved in steroidogenesis. A significant down-regulation of *hsd17ß3* was observed in the whole body. This may suggest a reduced formation of the enzyme, and in turn, reduction of testosterone synthesis. Additionally, expression of *cyp19b* was significantly up-regulated in the brain. Estrogens enhance the expression of *cyp19* in fish, in particular *cyp19b* in the brain (Callard et al., 2001), but also androgens differentially affect *cyp19* expression (Govoroun et al., 2001; Mouriec et al., 2009). This infers that EHMC acts on the expression of genes important for sex hormone synthesis. Forthcoming studies are needed to evaluate the consequences of observed transcriptional changes on plasma sex steroid levels, and on fertility and reproduction.

5.4.5 Conclusions

Our microarray study demonstrates that the expression profile in whole fish after EHMC-exposure affects many biological processes with pathways mainly involved in tissue remodelling, immune system response, inflammatory response, DNA-damage and apoptosis. The high abundance of significantly altered transcripts by EHMC makes a pathway analysis interpretation challenging. No single pathway or a simply defined pattern of affected cellular mechanisms could be identified. The whole-body microarray analysis approach captures the total sum of the transcriptional changes, hence providing an overall expression profile. As it is less sensitive than the organ-specific analysis, data interpretation is more challenging. For obtaining a more complete toxicological profile of a compound, we conclude that the analysis should cover multiple tissues.

Based on previous knowledge about hormonal activities of EHMC (Inui et al. 2003, Kunz and Fent 2006) we focused on hormone and related pathways (lipid and cholesterol metabolism, thyroid hormone biosynthesis or metabolism). We found that EHMC induces transcriptional chances of genes involved in hormone pathways and steroidogenesis. A down-regulation of *vtg1* in whole-body, brain and testis, but a dose-related up-regulation was observed in the liver. The induction of estrogen receptors *esr1* and *esr2b* in the whole body and of *vtg1*, *igfbp1*, and *esr2b* in the liver suggests an estrogenic activity of EHMC. The associated down-

regulation of ar in the liver seems to reflect an additional anti-androgenic activity. Differently, in the brain, a down-regulation of *vtg1*, *esr1*, *esr2b* and *rbp2a* suggests an anti-estrogenic, and the up-regulation of ar and *cyp19b* a potential androgenic activity. Therefore, the observed multiple hormonal activities of EHMC on gene expression in different tissues coincide with the activities *in vitro* (Kunz and Fent, 2006a). Differential expression profiles in the whole body and analysed tissues (brain, liver, testis) for several genes underlines the need for analysing multi-tissue gene profiling for understanding potential effects of this UV-filter. The data lead to the conclusion that EHMC has an overall low, but multiple hormonal activity. Furthermore, additional biological processes are modulated by EHMC, including tissue remodelling, cell differentiation, immune system response, DNA damage response and apoptosis.

The transcriptional changes were observed at environmentally realistic concentrations of 2.2 µg/L EHMC. Up to 19 and 3 µg/L EHMC were reported in raw wastewater (Balmer et al., 2005) and in a lake (Rodil et al., 2009), respectively. Forthcoming studies should investigate the toxicological consequences and environmental risks of EHMC, in particular by linking expressional changes with physiological outcomes including fertility and reproduction.

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Chapter 6

General Discussion and Conclusions

6.1 General discussion

The results achieved in the different studies of this thesis were discussed in detail in chapter two to five. This chapter provides a more general summary and the final discussion of all results obtained. We will highlight our results with relevance to the field of ecotoxicology and in environmental risk assessment of xenobiotics.

The present thesis focuses on the effects of human pharmaceuticals (diazepam and PKC412) and personal care products (UV-filter EHMC) on fish. PPCPs may be of concern as some of them can be active at extremely low concentrations (e.g. synthetic steroid hormones), may accumulate in organisms, or have unknown adverse effects. They have widespread use and are continuously released into the environment, so that concentrations in the range of ng/L to µg/L can be found in surface water. They can also have unpredictable biochemical interactions in mixtures and are able to bio-magnify in the food chain. Pharmaceuticals in particular are designed to interact with cellular receptors at low therapeutic doses and elicit specific biological effects. Since many target receptors have been conserved during evolution, pharmaceuticals may have a similar mode of action, and therefore evoke similar effects in fish to humans (Jones et al., 2007; Gunnarsson et al., 2008; Kostich and Lazorchak, 2008; Christen et al., 2010). Furthermore, other PPCPs such as cosmetic additives may have unexpected effects on lower organisms, due to their differences in targets and metabolism compared to mammals.

In addition to acute effects, studies in ecotoxicology also focus on chronic toxicity endpoints including biochemical and physiological effects, and effects on early life stages. Chemicals that are suspected of interfering with the hormonal system are investigated with respect to the effects on vitellogenin induction, fecundity, secondary sex-characteristics and reproduction. In addition, research has been recently performed focusing on expression changes of genes belonging to the detoxification system (phase I and phase II), hormonal pathways, stress response etc (Evrard et al., 2010; Li et al., 2010). However, there have been very few studies performed which try to link gene expression changes to ecologically more relevant effects such as behavioural alterations, adverse effects on reproduction, the ability to find food or to escape from predators. As only little is known about such correlations, they need investigating in more detail.

This thesis basically consists of two different parts. In the first part, a study is conducted to optimize a cellular assay for rapid measuring CYP3A enzyme activity changes. The up- or down-regulation of CYP3A enzyme activity may be an indication for exposure to PPCPs.

This enzyme plays a crucial role in detoxification processes and in the degradation of foreign substances (Guengerich, 1992; Livingstone, 1998; Hasler, 1999).

In the second part, several studies were performed to investigate correlations between gene expression changes and physiological changes induced by PPCPs in fish. Zebrafish at different developmental stages were exposed to two different pharmaceuticals (diazepam and PKC412) or to the UV-filter EHMC, respectively. Effects on the gene expression patterns were determined and then linked to physiological effects such as locomotor activity or effects on different spermatogenic stages. The hypothesis was thereby tested, whether alterations in gene expression are more sensitive than physiological effects, and whether gene expression alterations can be linked to physiological effects. In the following section, a short summary of the obtained results together with the most important conclusions are given.

Alteration of CYP3A activity *in vitro*

We modified a human whole cell assay to measure CYP3A enzyme activity in fish. We used 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as a substrate, which is known to be metabolized by the human CYP3A4 into a measurable fluorescent product. The basal catalytic activity in PLHC-1 and FHM cells is found to be higher than in human cells. In addition, it was demonstrated that this assay is capable of analysing for CYP3A induction by rifampicin, which is a known human CYP3A inducer, as well as inhibition by diazepam, a human CYP3A inhibitor.

Our findings have shown that BFC also acts as a substrate for fish CYP3A. The overall advantage of this optimised assay is that it can be performed on microtiter plates. This facilitates the screening for CYP3A inducers and inhibitors, as it is possible to simultaneously investigate different compounds and different concentrations on the same plate. However, there are also some critical aspects to be considered. BFC is not a sole substrate catalysed by CYP3A, but is found to be a substrate for CYP1A in mammals (Renwick et al., 2001). Therefore, for each compound an additional EROD assay (measures CYP1A activity) is necessary as a control. Furthermore, the CYP3A assay is not very sensitive as enzyme activity can only be detected at very high concentrations. This should be taken into consideration when the assay is applied as a first screening step for CYP3A inducers or inhibitors in environmental risk assessment.

Effects of diazepam on different developmental stages in zebrafish

This study focused on investigating the modes of action of diazepam in different developmental stages in zebrafish. We exposed adult males over 14 d to two different concentrations of diazepam (nominal 273 ng/L and 273 µg/L). In addition, zebrafish eleuthero-embryos were exposed for 5 dpf to the same diazepam concentrations. Gene expression changes were obtained by performing a microarray analysis with brain samples of adult males. Mainly genes belonging to the circadian rhythm were significantly altered. This alteration was also found in eleuthero-embryos. We attempted to link changes of the gene expression pattern with those on physiological endpoints. In the process the locomotor activity of adults and eleuthero-embryos were measured after 14 d or 6 dpf of exposure, respectively. However, only at the highest concentration is the locomotor activity significantly affected in eleuthero-embryos. In addition to behavioural changes, we performed histological analysis of seminferous tubuli in the testes to analyse different spermatogenic stages. However, a tendency towards an altered pattern of the different stages of sperm is only observed at the highest concentration, which is characterized by the occurrence of more interstitial tissue.

Diazepam binds on the $GABA_A$ receptor, which is the basic mode of action of these pharmaceutical eliciting effects on the CNS. Additionally, we have shown that this compound affects gene expression in the brain. The transcriptional response to diazepam at concentrations of 235 ng/L and 292 µg/L in zebrafish seems to be similar to that found in mammals (Akiyama et al., 1999; Carpentieri et al., 2006; Nishii et al., 2006; Novak et al., 2006). Genes belonging to the circadian rhythm, which are also altered in mammals (Akiyama et al., 1999; Carpentieri et al., 2006; Nishii et al., 2006; Novak et al., 2006), were significantly changed in fish. In addition, the swimming behaviour of eleuthero-embryos was significantly altered at 273 µg/L. No significant histological effects were observed in male testes, although changes in interstitial tissue were noted. In contrast to transcriptional changes occurring at both concentrations, physiological alterations were only observed at nominal 273 µg/L diazepam in eleuthero-embryos. This indicates that alterations in gene expression are more sensitive than the determined classical ecotoxicological endpoints. However, the lack of behavioural and physiological changes at 235 ng/L diazepam raises the question of whether and at which concentrations gene expression changes propagate to toxicological relevant measures at the organism level (that may affect even populations). In conclusion, this study confirms that important data are obtained by microarrays and qRT-PCR for identifying molecular effects and modes of action of pharmaceuticals that occur in the environment. However, such studies should be paralleled by classical ecotoxicological

tests to obtain effects on organisms relevant for populations, especially at environmentally relevant concentrations.

Effects of PKC412 on zebrafish eleuthero-embryos

Similar to the diazepam study, we tested the hypothesis that gene expression alteration is a more sensitive parameter than physiological parameters, but that they are linked with each other. In this study a microarray analysis was also performed. Zebrafish eleuthero-embryos are exposed for 6 dpf at two different concentrations of PKC412 (1.6 µg/L and 21 µg/L), which is a novel antineoplastic agent, not yet on the market. The aim of this study was the analysis of a novel compound prior to market release in order to obtain information about the ecotoxicological potential at concentrations expected to occur in the environment after it comes into use. This study is in contrast to the diazepam study where environmental concentrations are already known. The data obtained on gene expression changes were difficult to interpret as PKC412 is a non-selective kinase inhibitor with a broad spectrum. Again an alteration of circadian rhythm genes was observed, as kinases were also expressed in the hypothalamic suprachiastic nuclei. However, PKC412 had no significant effect on the locomotor activity in our experimental set-up. One reason could be that an observation over 2 h is too short to obtain significant effects. The time-points when measurements took place could also induce differences in behaviour. We performed our measurements in daylight, and effects would have probably been more pronounced at night. Additionally, effects on vascular development, appearance of apoptotic cells, DNA damage and formation of reactive oxygen species were further investigated. PKC412 had neither any influence on the vascular development in transgenic zebrafish eleuthero-embryos nor on the formation of reactive oxygen species. In contrast, apoptosis occurred on cells of the olfactory placodes and DNA damage was induced even to some extent at the 1.6 ug/L PKC412.

The overall conclusion of this study is the same as for the diazepam study. We demonstrate that the response of zebrafish eleuthero-embryos to different PKC412 concentrations was partly similar to the response in humans. Also here, alteration of gene expression was a more sensitive parameter than the measured physiological parameters. However, this study identified an additional challenge of microarray studies, the abundance of altered genes. Due to the broad kinase inhibition spectrum of PKC412, many genes were altered and therefore it was a challenge to identify a clear mode of action of this compound. This was in contrast to the findings with diazepam, where a clear effect pattern on the circadian rhythm was discovered. To obtain more precise insights into the mode of action of PKC412 in zebrafish, additional experiments on protein and physiological levels are indispensable. Furthermore, this study also raises the question as to what concentration the gene

expression changes propagate to significant changes at organism or even at population level. Therefore although gene expression studies are a novel and challenging tool for elucidation of molecular effects and even mechanisms of action, they are not sufficient for ecotoxicological risk assessment of pharmaceuticals. Therefore they have to be paralleled with physiological measures analyzed in classical tests.

Hormonal activity of UV-filter EHMC in zebrafish

Our interest in the following study focused on gene expression changes in zebrafish after exposure to the UV-filter EHMC, which belongs to one of the most frequently used compounds in sunscreens (Hauri, 2009). EHMC is found in the aquatic environment (Balmer et al., 2005), and accumulates in biota (Fent et al., 2010). The aim was to investigate molecular effects and the mode of action of EHMC.

In this study, adult male zebrafish were exposed over 14 d to nominal concentrations of 2.2 µg/L and 890 µg/L EHMC. The lower concentration was selected on the basis of environmental residue levels (Balmer et al., 2005), while the higher was selected in order to stimulate mRNA expression of genes to elucidate molecular modes of action of EHMC. Microarray analyses performed on small vertebrates such as zebrafish is a strategy for potentially obtaining a large amount of *in vivo* data on the transcriptome of the entire adult organism (Lam et al., 2008). Additionally, qRT-PCR was performed in the brain, liver and testes to compare tissue-specific expression patterns with those of whole body obtained with microarrays.

The expression profile in the whole body displays complex molecular effects, including multiple hormonal activities. This is reflected by the down-regulation of *vtg1*, which was not the case for *vtg3*, although the same trend was observed. This suggests an anti-estrogenic activity. Estrogens alter the expression of both *vtg1* and *vtg3* in fish (Miracle et al., 2006; Martyniuk et al., 2007). Similar to EHMC, the effect on *vtg1* expression was more pronounced than on *vtg3* expression (Martyniuk et al., 2007; Meng et al., 2010). In contrast, the induction of *esr1*, *esr2b* and *hsd17ß3* points to an estrogenic activity.

In the different tissues, EHMC displayed multiple effects on the expression of target genes, determined by qRT-PCR. An up-regulation of *vtg1*, *igfbp1*, and *esr2b* was observed in the liver, which is in accordance to a potential estrogenic activity of EHMC (Palmer and Palmer, 1995; Kloas et al., 1999). The associated down-regulation of *ar* may either reflect an estrogenic activity (due to feedback regulation), or an additional anti-androgenic activity. (Filby et al., 2007) found no modulation of the *ar* transcript after EE2 exposure in male

fathead minnows, while a strong down-regulation in hepatic *ar* mRNA was observed after exposure to the anti-androgen flutamide.

However, in the brain, a down-regulation of *vtg1*, *esr1 and esr2, rbp2* and an up-regulation of *ar* was observed, which is in accordance to a potential anti-estrogenic and androgenic activity. In testes, with the exception of *gadd45b* transcript, the same trend was observed for target-genes selected from microarray results. The differential effect of EHMC on *vtg1* expression in different tissues highlights the importance for analyzing transcription profiles in multiple tissues to identify molecular effects and mode of actions. However, for the testes no clear dose-response-relationship upon EHMC exposure was observed. In fact, in testes we observed the highest variability in terms of Ct value and the lowest responsiveness among the selected genes. In agreement with the pathways involved in apoptosis, growth arrest and DNA-damage-inducible beta (*gadd45b*) was significantly up-regulated in whole body, in particular at 2.2 µg/L EHMC. This gene, which is involved in regulation of growth and apoptosis, was significantly up-regulated after EE2 exposure in zebrafish liver (Hoffmann et al., 2006). However, this was not the case in our study.

EHMC-exposure also affected the expression of genes involved in steroidogenesis. A significant down-regulation of *hsd17ß3* was observed in the whole body. This may suggest a reduced formation of the enzyme*,* and in turn, reduction of testosterone synthesis. Additionally, expression of *cyp19b* was significantly up-regulated in the brain. Estrogens enhance the expression of *cyp19* in fish, in particular *cyp19b* in the brain (Callard et al., 2001), but also androgens differentially affect *cyp19* expression (Govoroun et al., 2001; Mouriec et al., 2009). This infers that EHMC acts on the expression of genes important for sex hormone synthesis.

In addition, Gene Ontology (GO) analysis was performed to identify functional groups of genes of interest. At both concentrations differentially altered genes fell into 1637 different categories. The many GO terms affected by EHMC exposure are general descriptions, which based on the results of this experiment alone, makes it difficult to link them to a specific mode of action. Furthermore, we performed a pathway analysis with MetaCore. The different treatments had 33 (2.2 µg/L EHMC: 33 maps; 890 µg/L EHMC: 36 maps) maps in common. No single pathway or a simply defined pattern of affected cellular mechanisms could be identified. On the contrary, the affected pathways are numerous and include those belonging to tissue remodelling, cell differentiation, immune system response, DNA-damage response and apoptosis, to name the most significant ones. Taken together, our data support the hypothesis of an endocrine activity of EHMC.

This study demonstrates that the expression profile after EHMC treatment is tissue-specific and varies between different organs. In contrast, the whole body gene expression analysis captures the total sum of the transcriptional changes in the entire organism as a single entity. This gives an overall gene expression profile of the compound, but has limitations such as loss of weak signals from smaller tissues (tissue-specific location of the response cannot be achieved) and in particular do not provide a clear cut-off of altered genes and consequently pathways. Therefore, even if the whole-body analysis provides an overall picture on altered genes, it is less sensitive and, as consequence, data interpretation is much more challenging than investigating a single tissue alone, such as liver. On the other hand, focusing on a single organ does not allow one to obtain a more complete feature of the effects determined from a weak study compound such as EHMC. Therefore, for obtaining a more complete toxicological profile of the compound, the analysis should cover multiple tissues.

When using whole-body microarrays the most influential tissue for the response remains undefined. In case of EHMC, data from the whole body (microarray) mainly reflected the results obtained by qRT-PCR in the brain (and to a lesser extent those obtained in testes), but interestingly, not those of the liver, which is often analysed (Pesonen and Andersson, 1991; Hinton and Couch, 1998; Hinton, 2000). This demonstrates that transcriptional effects of chemicals should be analysed by a multiple organ approach, and not by one tissue alone.

The expression profile in the whole body displays a complex molecular effects profile. The abundance of significantly altered genes by EHMC is even higher than in case of PKC412 (Chapter 4). EHMC affects the transcription of genes involved in hormonal pathways and steroidogenesis, pointing to multiple hormonal activities of this UV-filter, which is in accordance to *in vitro* activities (Kunz and Fent, 2006). The transcriptional changes involve a large number of genes and pathways. This makes a pathway analysis interpretation of EHMC even more complicated, and therefore, no single pathway or a clearly defined pattern of affected cellular mechanisms could be identified, as in case of diazepam (Chapter 3). On the contrary, the affected pathways are numerous and include - among others - those belonging to tissue remodelling, cell differentiation, immune system response, DNA-damage response and apoptosis.

Additionally, this study also points out the need for a correlation of the transcriptional changes with physiological effects in order to interpret relevant toxicological impacts on the organism. One should always keep in mind that gene expression changes do not necessarily translate to physiological changes, as there are several modification steps from the mRNA to

the protein and to the physiological outcome. Therefore, forthcoming studies are needed to show whether EHMC affects plasma sex steroid levels and consequently, fertility and reproduction in adult zebrafish.

To summarize the experiments on two human pharmaceuticals and the UV-filter EHMC, it was shown that they can cause adverse effects on gene expression and physiology at concentrations not far from worst-case environmental concentrations. The major advantage of the transcriptional analysis is the identification of possible mechanisms of action of a given compound. However, in most cases gene expression alteration gives some preliminary indications about which physiological effects are compound-induced. There are several modification steps from the gene to the protein, and therefore gene expression changes do not necessarily affect protein expression or other physiological processes. Nevertheless, such analyses offer a powerful tool of identifying underlying molecular mechanisms of toxicological effects and possibly, for screening for new biomarkers.

It has to be borne in mind that physiological effects can have their limitations. As the studies are conducted in the laboratory and not in the field, they do not exactly reflect the environmental situation. As a consequence, extrapolation from the laboratory to the field should take this limitation into consideration. However, the results obtained are nevertheless a good indication of what can happen in the environment.

To obtain mechanistic information on molecular effects and the toxic modes of action of PPCPs, microarray analysis offers great potential. Nevertheless, there is the necessity of correlating effects obtained at gene expression level with effects at higher levels of biological organization. In our studies we have partly shown that gene expression changes can be linked to ecologically more relevant effects such as swimming behaviour (diazepam) or apoptosis (PKC412).

In summary, based on transcription profiles and associated physiological effects, this thesis has given new insights into the modes of action of two different pharmaceuticals and the UV-filter EHMC on fish. Our findings that gene expression changes could partly be linked to physiological effect are promising for further research on the mode of action of environmentally relevant PPCPs.

More research is needed in the future on the linkage between gene expression and physiological effects, in order to assess the value of transcription profiling in ecotoxicology. As gene expression analysis is not a standardized procedure yet, further validation is needed

with a broader set of compound classes, and with respect to the correlation with physiological effects. It should be pointed out that further research is needed to investigate the value of gene expression changes for environmental risk assessment. The ultimate aim of using this approach will be to gain more important information and a better focus on the molecular modes of action of compounds for application in environmental risk assessment, complementing already existing test systems (i.e. OECD guidelines).

In case of diazepam, significant physiological changes of single compounds are only found at concentrations that were up to 10,000 times higher than environmental concentrations. However, in the environment, different compounds occur not as single entities, but in mixtures of different compounds. Mixtures of the same substance class often act on the same target receptor and usually exhibit an additive nature (Kunz and Fent, 2009). Therefore, a compound can supplement other similar substances in a mixture resulting in a higher net effect. PPCPs should also be analysed in mixtures to evaluate whether they exhibit physiological effects at environmentally relevant concentrations. These considerations could be of importance for environmental risk assessment. Knowledge about the potential toxicological modes of actions and associated hazards of PPCPs ultimately leads to an improvement in environmentally safe handling of these substances, their environmental management, and consequently, to limited harm to the aquatic environment.

6.2 Outlook

This thesis reveals new insight towards different modes of action of PPCPs in fish. Our research mainly focused on the investigation of gene expression patterns upon exposure to PPCPs and their linkage to physiological effects.

Effects on gene expression were mostly found at concentrations near environmentally relevant concentrations and higher. In contrast, physiological effects mainly occurred at concentrations that were significantly higher than environmental concentrations, indicating that transcription profiles represent a more sensitive toxicological parameter.

In consequence, the following questions should be addressed for further studies:

- Are there similar gene expression patterns that are shared by individual PPCPs belonging to the same substance class? And if so, is there a specific and common expression pattern that allows the identification of this class of compounds, and the possibility of predicting them for novel similar compounds?
- To what extent can gene expression patterns be correlated to physiological effects, and is there a concentration range where a correlation exists?

- What are the effects of PPCPs mixtures on gene expression and on higher levels of biological organization?
- Can toxicogenomic approaches be used for analysing the modes of action of environmental chemicals? Is a standardisation possible for using the results for environmental risk assessment?
- What is the value of gene expression changes for assessing the ecotoxicologcial potential of compounds and for their environmental risk assessment?

In order to answer some of these questions, several general points should be regarded. Before starting a microarray analysis and obtaining a broad spectrum of information, the goal of the project should be clearly formulated and it should be defined for what purpose the microarray data will be used. Depending on the goal, it is better to perform a whole body analysis to obtain a more general and overall transcriptional profile, instead of achieving specific tissue-specific information. This could be a good approach for analysing compounds whose mode of action is not clear, and if a detailed mechanistic study is not needed. However, to address more specific questions on the toxicological profile of a compound, the analysis of single tissues is more appropriate, as demonstrated in this thesis for diazepam. The selection of the appropriate tissue might be difficult, but can be based on known information from mammals. If no such information exists, as in the case of the UV-filter EHMC, a multiple-organ approach is more appropriate. Analysis of single tissues has also the advantage that the abundance of altered genes is lower, and therefore, an interpretation becomes more reliable and comprehensible.

Up to now, most analytical tools for evaluating microarray data, such as MetaCore pathway analysis, are designed for human purposes only. However, for many genes in zebrafish or fathead minnows there are no human orthologues. Therefore, the possibility of losing information that could be otherwise important for the interpretation of the whole data set, is often present. Potentially, a solution would be to start generating special zebrafish maps with available data found in literature; however, this is a time-consuming procedure.

To obtain a more general gene expression pattern specific for compounds of a given chemical class, a set of chemicals should be tested. The data set may then offer the possibility of defining prediction models for this kind of chemical class, with the assumption that substances with similar mechanistic effects or modes of action share similar biological and toxicological responses. The gene expression pattern of a novel compound or mixture can then be compared with that of the model, and possibly, predictions about its mode of action and toxicity can be made.

In addition to pathway analysis and possible prediction models, an attempt should be made to link gene alteration patterns with physiological and biological effects. In order to restrict the transcription profile to a core set of genes, proteomics would be a helpful technique, thus allowing restriction of the expression pattern to those genes where the corresponding protein is also altered.

In addition, it is important that not only high concentrations of a compound are tested, but also environmentally relevant concentrations. Linking physiological and biological effects to gene expression patterns at low concentrations is more significant than evaluating high concentrations alone for environmental risk assessment, because this reflects better the situation in the environment.

6.3 References

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Appendix 1

Table 1

Significantly altered genes in the brain of male zebrafish after exposure to 235 ng/L and 291 µg/L diazepam (adjusted p<0.05)

			Fold change (log_2)	
Agilent ID	**Sequence Accession #**	**Gene name**	**235 ng/L**	**291 µg/L**
A_15_P100260	NM_131790	cryptochrome 1b	2.51	-3.00
A_15_P100831	NM_212679	basic helix-loop-helix domain containing, class B, 2		-3.11
A_15_P100884	NM_131787	cryptochrome 4	1.55	2.04
A_15_P101039	NM_131577	aryl hydrocarbon receptor nuclear translocator-like 1a	2.19	2.67
A_15_P102608	TC338072		2.48	-3.36
A_15_P103210	NM_200692	N-myc downstream regulated gene 1, like	1.78	3.97
A_15_P103364	NM_131584	period homolog 3 (Drosophila)	-3.29	-7.88
A_15_P103946	NM_131788	cryptochrome 5	-3.56	-4.27
A_15_P104122	NM_001017652	synaptotagmin XIII (syt13), zgc:112063	-1.53	-2.17
A_15_P104177	NM_200364	hydroxysteroid (17-beta) dehydrogenase 3	-1.59	-2.32
A_15_P104502	NM_212439	period homolog 4	-3.78	-6.52
A_15_P105008	NM_201064	zgc:66475	-1.62	-2.01
A_15_P105258	NM_001113589	similar to Hsp70 protein, LOC560210	-1.61	-3.40
A_15_P105750	XM_001335424	hypothetical protein LOC797197		2.77
A_15_P106879	NM_200792	arrestin 3, retinal (X-arrestin)	-2.48	-4.42
A_15_P108054	NM_131400	thyrotroph embryonic factor	-1.88	-2.40
A_15_P109547	TC346523		2.32	2.19
A_15_P110290	NM_205729	nuclear receptor subfamily 1, group d, member 1	-6.28	-3.14
A_15_P110618	ENSDART00000010477		-1.46	-2.71
A_15_P111207	ENSDART00000043507	si:ch211-284a13.1	-9.09	-8.71
A_15_P111741	XM_001337603	similar to Wu:fk63e10 protein	5.46	8.25
A_15_P112116	XM_679130	hypothetical LOC572734	1.49	2.10
A_15_P113877	NM_201158	Danio rerio aldehyde dehydrogenase 4 family, member A1 (aldh4a1), nuclear gene encoding mitochondrial protein, mRNAzgc:63592	-1.56	-2.18
A_15_P114878	TC322430		-1.87	-2.72
A_15_P115546	NM_178300	aryl hydrocarbon receptor nuclear translocator-like 1b	2.44	3.17
A_15_P116826	NM_131867	heat shock factor 2	-2.68	-3.04
A_15_P117222	XR_029365	similar to RCE1 homolog, prenyl protein peptidase		-2.65
A_15_P118405	XM_688726	hypothetical LOC565446	-1.50	-2.09
A_15_P118684	ENSDART00000006474	glycine receptor, alpha 4b	1.67	2.24
A_15_P119540	CK015579	wu:fj84d10	-2.61	-2.86
A_15_P120707	NM_205686	cryptochrome DASH	-3.21	-3.91
A_15_P120925	NM_201056	MAX dimerization protein 3	2.01	2.91

A_15_P121171	BC066388	aryl hydrocarbon receptor nuclear translocator-like 2	2.65	5.57
A_15_P130406	NM_001014369	si:dkey-31f5.7	1.91	2.54
A_15_P133821	BC060943	wu:fk63e10	5.88	9.25
A_15_P137031	ENSDART00000073440	similar to DnaJ-like subfamily A member 4	-1.58	-2.58
A_15_P137886	NM_131787	cryptochrome 4	1.58	2.15
A_15_P141096	NM_001039107	basic helix-loop-helix domain containing, class B, 3 like	-2.30	-3.55
A_15_P143946	ENSDART00000041615	UDP glucuronosyltransferase 5 family, polypeptide G1	2.07	2.82
A_15_P144496	ENSDART00000075588	similar to MGC115669 protein	-2.42	-2.78
A_15_P144761	ENSDART00000083569	ornithine decarboxylase antizyme 2, like	-1.35	-2.00
A_15_P144886	NM_212439	period homolog 4	-3.09	-5.23
A_15_P147921	NM_001004120	nuclear factor, interleukin 3 regulated	2.08	2.54
A_15_P150786	NM_199611	arginase, type II	-2.05	-3.18
A_15_P151176	NM_001082936	cytochrome P450, family 2, subfamily J, polypeptide 29		-2.54
A_15_P153641	NM_200914	abhydrolase domain containing 2	-1.81	-2.23
A_15_P159451	NM_001014369	si:dkey-31f5.7	2.02	2.85
A_15_P160171	ENSDART00000082475	Rev-erbgamma-B	2.36	10.49
A_15_P161566	NM_131786	cryptochrome 3	-2.06	-2.77
A_15_P166596	NM_131400	thyrotroph embryonic factor	-1.92	-2.46
A_15_P168101	NM_131790	cryptochrome 1b	-2.54	-3.18
A_15_P170926	NM_001014369	si:dkey-31f5.7	2.13	2.78
A_15_P173191	NM_131792	cryptochrome 2b	1.67	3.26
A_15_P175566	NM_131578	aryl hydrocarbon receptor nuclear translocator-like 2	2.64	5.40
A_15_P176961	BC097065		1.92	2.34
A_15_P177056	ENSDART00000043507	si:ch211-284a13.1	-10.81	-9.57
A_15_P180171	ENSDART00000077084	similar to LOC553272 protein	1.41	2.55
A_15_P183846	ENSDART00000103169	Rev-erbgamma-A	1.55	4.43
A_15_P186116	ENSDART00000023506	similar to Solute carrier family 16 (monocarboxylic acid transporters), member 1	-1.71	-2.57
A_15_P186256	ENSDART00000103263	similar to cysteinyl leukotriene receptor 1	-1.95	-5.15
A_15_P187316	NM_205729	nuclear receptor subfamily 1, group d, member 1	-5.94	-2.77
A_15_P188666	ENSDART00000040156	hypothetical protein LOC793064	1.59	2.07
A_15_P191501	ENSDART00000012376	gamma-aminobutyric acid (GABA) receptor, rho 1	-2.02	-4.12
A_15_P191926	*ENSDART00000013140*		4.77	7.38
A_15_P194301	NM_001077731	zgc:154093	-1.39	-2.44
A_15_P196411	NM_200692	N-myc downstream regulated gene 1, like	1.66	3.05
A_15_P198036	NM_001111164	encephalopsin	2.29	3.28
A_15_P200426	ENSDART00000076153	hypothetical LOC559531	1.57	2.01
A_15_P204936	NM_131400	thyrotroph embryonic factor	-1.94	-2.25

A_15_P206011	NM_001045115	si:ch211-63o20.5	-3.13	-3.41
A_15_P206101	NM_001045055	si:ch211-132b12.7	-5.07	-8.68
A_15_P210616	NM_001017753	zgc:112094	-2.0	-2.2
A_15_P212226	BC090493	similar to IMP (inosine monophosphate) dehydrogenase 1	2.01	2.61
A_15_P212631	BC134075		-6.26	-17.51
A_15_P236266	NM_001030183	period homolog 1 (Drosophila)	-5.83	-4.48
A_15_P243801	XM_692570	hypothetical LOC569202	1.40	2.49
A_15_P262641	AI965034		1.46	2.03
A_15_P263411	BC096780	cryptochrome 2b	1.67	3.00
A_15_P265011	XM_001340943	hypothetical protein LOC100000872	-1.47	-2.41
A_15_P265381	XM_689253	pleckstrin homology domain containing, family G (with RhoGef domain) member 2	1.52	2.25
A_15_P280791	TC344055		1.99	3.92
A_15_P280961	TC344117		-3.48	-4.54
A_15_P281421	TC344277		2.36	6.27
A_15_P290916	XM_001332002	similar to Wu:fk63e10 protein	5.99	11.31
A_15_P297801	CA475016		-1.96	-2.48
A_15_P348800	TC333899		1.30	2.03
A_15_P358610	CT662681		1.70	2.72
A_15_P359680	CN168295		-6.86	-12.31
A_15_P378335	ENSDART00000041615	UDP glucuronosyltransferase 5 family, polypeptide G1	2.12	3.18
A_15_P382570	TC356482		-2.36	-4.18
A_15_P394380	EL651135		5.93	10.54
A_15_P433820	BC131854	basic helix-loop-helix domain containing, class B, 3 like	-2.29	-3.49
A_15_P465930	TC353762		-1.74	-2.85
A_15_P477630	XM_691681	hypothetical LOC568355	-1.98	-2.87
A_15_P509447	ENSDART00000097466		-1.65	-2.10
A_15_P509602	CT670730		-2.87	-6.46
A_15_P519787	CT670730		-2.27	-4.98
A_15_P520947	CK236112		2.49	5.84
A_15_P544952	NM_200061	arachidonate 5-lipoxygenase-activating protein	-1.68	-2.15
A_15_P551002	XM_694484	hypothetical LOC570947	1.67	2.33
A_15_P561512	TC336646		1.73	3.43
A_15_P567227	NM_001083013	tensin 3	-1.50	-2.41
A_15_P597722	CT652640		-1.86	-2.01

Appendix 2

Table 1

Significantly altered genes in zebrafish eleuthero-embryo after exposure to 1.3 µg/L and 21 µg/L PKC412 (adjusted p<0.05).

Agilent ID	Sequence Accession #	Gene name	Fold change (log_2)	
			1.3 µg/L	21 µg/L
A_15_P100036	BC124598		1.52	2.24
A_15_P100059	NM_001020482	carboxypeptidase A1 (pancreatic)	2.89	-
A_15_P100260	NM_131790	cryptochrome 1b	-2.08	-1.54
A_15_P100294	XM_692376	hypothetical LOC569014	-1.84	-2.36
A_15_P100312	NM_001013276	crystallin, gamma S1	-	2.20
A_15_P100495	NM_001001843	tryptophan hydroxylase 1, like	-	-2.19
A_15_P100560	NM_001045235	zgc:136815	2.04	-
A_15_P100658	TC316008		-	4.30
A_15_P100666	NM_131190	serum response factor	-	2.03
A_15_P100836	BQ480828		1.63	2.32
A_15_P100839	NM_001007054	zgc:92590	2.61	-
A_15_P100919	EH535115	wu:fb48d04	1.69	2.21
A_15_P101039	NM_131577	aryl hydrocarbon receptor nuclear translocator-like 1a	2.5	3.12
A_15_P101064	ENSDART00000043658	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	-2.10	-
A_15_P101201	ENSDART00000043434	zgc:103728	-	4.04
A_15_P101305	TC334332		3.19	6.28
A_15_P101403	BM071654		1.58	2.58
A_15_P101676	NM_001003426	zgc:92745	3.41	-
A_15_P101734	NM_001020795	crystallin, gamma M7	-	2.08
A_15_P101959	BM571381		-	2.05
A_15_P102163	NM_200100	hypoxia induced gene 1	-2.59	-1.82
A_15_P102261	NM_001003776	zgc:100982	-2.99	-1.70
A_15_P102367	NM_001080182	zgc:158752	-	-2.59
A_15_P102459	NM_182857	period homolog 2 (Drosophila)	-2.14	-3.08
A_15_P102468	NM_001110478	capthepsin B, b	-	-2.00
A_15_P102953	NM_199869	insulin induced gene 1	2.09	-
A_15_P102987	NM_001002567	zgc:92754	-	-2.16
A_15_P103003	NM_213050	zgc:56053	-	-2.11
A_15_P103094	NM_001044836	si:dkey-14d8.7	2.12	-
A_15_P103210	NM_200692	N-myc downstream regulated gene 1, like	3.16	4.26
A_15_P103395	NM_001045343	zgc:153037	-	-2.71
A_15_P103587	NM_200186	zgc:56005	-	-4.17
A_15_P103702	BC154648		2.09	-
A_15_P103714	NM_194393	guanylate cyclase activator 1C	-2.32	-1.50

A_15_P103716	NM_131870	guanylate cyclase activator 1A	-	-3.17
A_15_P103946	NM_131788	cryptochrome 5	-2.68	-2.86
A_15_P104101	BI428525	hypothetical LOC556853	-	-2.61
A_15_P104271	NM_001002674	zgc:92440	2.78	-
A_15_P104346	ENSDART00000099847		2.11	-
A_15_P104502	NM_212439	period homolog 4	-4.40	-3.17
A_15_P104513	NM_207052	midnolin	-	2.03
A_15_P104616	XM_677857	retinoic acid receptor, alpha b	-	2.76
A_15_P104624	NM_001007038	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	-2.03	-1.80
A_15_P104868	XM_001343386	hypothetical protein LOC100003999	-4.68	-6.94
A_15_P104936	BQ074590		-	2.10
A_15_P104960	NM_213130	adenylate kinase 3-like 1	-	-2.21
A_15_P105015	NM_199607	carboxyl ester lipase, tandem duplicate 1	3.86	-
A_15_P105032	EH605763	hemoglobin beta embryonic-3	-	2.20
A_15_P105154	NM_212950	parathyroid hormone 1a	-	3.04
A_15_P105312	NM_131372	invariant chain-like protein 2	2.12	-
A_15_P105715		angiopoietin 2	-	3.63
A_15_P105742	NM_001077744	zgc:152772	-3.63	-
A_15_P105955	NM_001007051	vesicle amine transport protein 1 homolog (T californica)	-2.07	-
A_15_P106368	NM_131814		-	2.18
A_15_P106535	TC306742		3.12	-
A_15_P106633	NM_001003737	zgc:92041	2.41	-
A_15_P106879	NM_200792	arrestin 3, retinal (X-arrestin)	-3.82	-3.49
A_15_P107148	NM_182891	opsin 1 (cone pigments), medium-wave-sensitive, 2	-	2.36
A_15_P107160	AA495045		-	2.14
A_15_P107533	NM_200871	phosphodiesterase 6C, cGMP-specific, cone, alpha prime	-2.24	-2.00
A_15_P107751	NM_182942	myxovirus (influenza) resistance A	-	2.96
A_15_P107809	XM_684946	hypothetical LOC561538	2.18	-
A_15_P107868	BC059655	zgc:73337	-	2.51
A_15_P108001	CK019211	wu:fb40b03	-	3.36
A_15_P108054	NM_131400	thyrotroph embryonic factor	-3.48	-3.09
A_15_P108174	NM_131581	odorant receptor, family D, subfamily 111, member 10	-	2.02
A_15_P108216	NM_001005394	glycophorin C (Gerbich blood group)	-	-2.15
A_15_P108469	ENSDART00000066833		2.13	-
A_15_P108688	NM_001001949	granulin a	2.21	-
A_15_P109076	CK707447		-	2.22
A_15_P109078	DT061329		-1.67	-2.44
A_15_P109206	CK363363		-	6.42
A_15_P109244	TC346477		-	2.18

A_15_P109267	TC316344		2.17	-
A_15_P109281	BI878336		-	2.71
A_15_P109547	TC346523		-	2.57
A_15_P109551	TC330139		1.75	2.63
A_15_P109660	NM_001008579	zgc:92239	-2.09	-1.79
A_15_P109678	NM_001020732	zgc:109977	-2.57	-1.94
A_15_P109683	NM_212999	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	1.86	2.86
A_15_P110098	NM_001007763	N-acylsphingosine amidohydrolase 2	-	-4.69
A_15_P110142	TC353878		-	2.59
A_15_P110191	BI673679		-	2.36
A_15_P110290	NM_205729	nuclear receptor subfamily 1, group d, member 1	-14.73	-7.41
A_15_P110367	ENSDART00000014183	glycosyltransferase 25 domain containing 2	-	2.11
A_15_P110417	ENSDART00000101764	similar to type IV antifreeze protein	-	3.15
A_15_P110650	NM_199733	zgc:56201	-	-2.77
A_15_P110860	NM_001004112	zgc:92375	-	-2.27
A_15_P111207	ENSDART00000043507	si:ch211-284a13.1	-7.04	-4.21
A_15_P111244	TC347468		2.20	3.54
A_15_P111724	CN500628		-2.42	-
A_15_P111741	XM_001337603	similar to Wu:fk63e10 protein	5.74	6.13
A_15_P111830	XM_686911	hypothetical LOC563546	-4.36	-3.43
A_15_P111831	NM_213196	zgc:77836	-	2.01
A_15_P112208	NM_001109850	zgc:173915	2.51	-
A_15_P112394	NM_198072	5-methyltetrahydrofolate-homocysteine methyltransferase	-1.69	-2.52
A_15_P112425	NM_131372	invariant chain-like protein 2	2.57	-
A_15_P112603	NM_131610	adenosine deaminase, RNA-specific, B1	-2.06	-1.47
A_15_P112700	NM_001002181	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	-2.04	-
A_15_P112731	NM_001077344	zgc:153787	2.64	-
A_15_P113163	ENSDART00000063484	hypothetical LOC568321	2.88	-
A_15_P113243	BM316038		-	2.37
A_15_P113391	BC142906		-2.05	-1.95
A_15_P113683	NM_001003777	zgc:100975	2.04	2.13
A_15_P113690	BC153933		-2.29	-1.64
A_15_P113950	ENSDART00000024615		2.07	4.93
A_15_P114484	AF246175	T cell receptor alpha constant	1.88	2.12
A_15_P114533	AI964281	progesterone receptor membrane component 1	-	2.33
A_15_P114542	NM_001083825	similar to Glutaminase, kidney isoform, mitochondrial precursor (GLS) (L-glutamine amidohydrolase) (K-glutaminase)	2.07	-
A_15_P114615	ENSDART00000024136	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	1.85	2.34
A_15_P114692	NM_201195	non-metastatic cells 4, protein expressed in	2.25	-

A_15_P114878	TC322430		-3.10	-3.73
A_15_P114960	NM_130957	clock	2.85	2.70
A_15_P115043	NM_131075	metallothionein	-	2.29
A_15_P115158	ENSDART00000066936	zgc:56123	2.26	-
A_15_P115437	NM_001017779	zgc:110366	-	-2.01
A_15_P115542	NM_130978	neurogenic differentiation	2.23	2.85
A_15_P115546	NM_178300	aryl hydrocarbon receptor nuclear translocator-like 1b	3.10	2.03
A_15_P115579	EH602155		-2.33	-
A_15_P115758	NM_001082930	si:ch211-240l19.8	2.74	-
A_15_P115764	NM_199848	adenosine monophosphate deaminase 3	2.35	1.80
A_15_P116070	ENSDART00000080859		-	2.10
A_15_P116148	NM_001003450	zgc:92511	2.03	-
A_15_P116208	NM_199271	carboxypeptidase A5	3.01	-
A_15_P116231	NM_001002868	dpy-19-like 1, like (H. sapiens)	1.77	2.42
A_15_P116346	NM_200160	zgc:55511	2.12	-
A_15_P116484	NM_001013478	si:dkey-13a21.4	2.44	-
A_15_P116818	CD606691		-	2.40
A_15_P116981	TC335896		2.72	1.63
A_15_P117021	NM_001083023	zgc:158846	2.15	-
A_15_P117046	NM_131253	opsin 1 (cone pigments), medium-wave-sensitive, 1	2.06	2.70
A_15_P117062	NM_001006039	zgc:103427	-2.39	-1.72
A_15_P117089	NM_001080176	zgc:158856	-	2.09
A_15_P117344	XM_678566	LOC555906	-2.02	-
A_15_P117376	BM184314		-	2.09
A_15_P117784	NM_199271	carboxypeptidase A5	3.61	-
A_15_P117864	NM_001024408	elastase 3 like	3.56	-
A_15_P118032	TC363214		2.72	3.18
A_15_P118042	ENSDART00000082372		1.98	2.23
A_15_P118079	NM_001007349	zgc:103473	-2.03	-2.03
A_15_P118514	NM_201077	zgc:63647	2.28	-
A_15_P118528	NM_200071	Rhesus blood group, B glycoprotein	-	-2.22
A_15_P118532	NM_001002217	zgc:91794	2.06	-
A_15_P118568	TC351820		-2.42	-2.34
A_15_P119281	NM_001002143	zgc:86722	-	-2.08
A_15_P119413	NM_131192	opsin 1 (cone pigments), short-wave-sensitive 2	1.82	2.15
A_15_P119429	NM_001002443	opsin 1 (cone pigments), long-wave-sensitive, 2	2.43	2.13
A_15_P119431	CN170396	ribosomal protein S26, like	-	2.56
A_15_P119515	NM_198984	purinergic receptor P2X, ligand-gated ion channel, 7	-	-2.99
A_15_P119523	BC142812		-4.00	-1.72

A_15_P119540	CK015579	wu:fj84d10	-5.05	-2.97
A_15_P119573	CK353894		-2.59	-
A_15_P119688	XM_690900	intermedin precursor	2.80	2.85
A_15_P119893	NM_001044861	si:ch211-200o3.4	-	-2.42
A_15_P119933	TC322484		-	2.27
A_15_P120064	CK017430		-	3.06
A_15_P120105	NM_131518	CD81 antigen	-	-2.67
A_15_P120127	NM_200610	zgc:63622	-	2.07
A_15_P120164	CK027491		-	2.27
A_15_P120402	TC346262		-2.32	-
A_15_P120615	XM_001332059	similar to prominin-like 2	-	2.61
A_15_P120653	ENSDART00000077511	LOC553366	2.12	-
A_15_P120707	NM_205686	cryptochrome DASH	-2.37	-2.06
A_15_P120767	NM_001007132	zgc:123068	-	3.28
A_15_P120802	BQ783861		-	2.35
A_15_P120815	ENSDART00000040598		3.81	-
A_15_P120831	NM_001037101	PASG	-2.00	-2.09
A_15_P120851	NM_001013526	six-cysteine containing astacin protease 3	2.25	-
A_15_P120925	NM_201056	MAX dimerization protein 3	-	2.28
A_15_P121032	ENSDART00000076523		2.31	1.41
A_15_P131306	DQ851840	PR domain containing 1b	2.3	2.88
A_15_P131706	ENSDART00000035967	bromodomain-containing 2a	-	2.34
A_15_P132596	AY050507	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	1.94	2.30
A_15_P132666	AY193826	nidogen 2 (osteonidogen)	-	-2.47
A_15_P133501	NM_212999	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	1.88	2.94
A_15_P133581	BC051151		-2.66	-
A_15_P133821	BC060943	wu:fk63e10	5.82	6.49
A_15_P134231	NM_001045210	zgc:113032	-2.92	-2.19
A_15_P134246	BC090504	si:ch211-219i10.1	-	-2.32
A_15_P135406	ENSDART00000023265	hypothetical protein LOC797548	-	2.42
A_15_P135701	ENSDART00000041988	similar to solute carrier family 34 (sodium phosphate), member 1	-	2.20
A_15_P136186	ENSDART00000035894	hypothetical LOC570421	1.57	2.94
A_15_P136411	ENSDART00000104449	hypothetical LOC571565	-	2.23
A_15_P136686	ENSDART00000103901		-	2.03
A_15_P136851	ENSDART00000103620	similar to Leucine rich repeat containing 56	-	2.78
A_15_P137851	ENSDART00000082490	similar to K+ channel tetramerization protein	2.18	1.48
A_15_P137856	ENSDART00000065377	hypothetical LOC570940	2.60	6.52
A_15_P137886	NM_131787	cryptochrome 4	2.29	1.80
A_15_P138221	ENSDART00000105618		-	2.32

A_15_P139056	ENSDART00000104909		-5.13	-2.81
A_15_P139066	ENSDART00000086936	si:ch211-239j15.1	-4.91	-3.15
A_15_P139526	NM_001024812	zgc:113944	1.74	2.15
A_15_P140252	NM_001024402	zgc:109991	1.58	-2.02
A_15_P141026	NM_001018121	gammaM2a-crystallin	-	2.36
A_15_P141096	NM_001039107	basic helix-loop-helix domain containing, class B, 3 like	-2.56	-2.63
A_15_P141136	NM_001020785	interleukin 10	2.41	-2.04
A_15_P141456	NM_001020788	interleukin 17c	-	2.55
A_15_P141526	NM_001102388	zgc:171642	-2.39	-2.38
A_15_P141596	NM_001020506	taste receptor, type 2, member 203	-	2.85
A_15_P142196	NM_001045376	zgc:153417	-2.35	-1.43
A_15_P143116	NM_001017825	zgc:110200	-	-2.15
A_15_P143801	NM_001103197	zfconnexin 41.04	2.28	-
A_15_P144356	ENSDART00000075014	similar to Autocrine motility factor receptor	2.23	1.63
A_15_P144496	ENSDART00000075588	similar to MGC115669 protein	-2.69	-2.17
A_15_P144641	ENSDART00000021013		1.97	2.18
A_15_P144801	ENSDART00000073932	hypothetical LOC560802	-2.48	-2.84
A_15_P144876	ENSDART00000073827	collagen, type IV, alpha 5 (Alport syndrome)	-	-2.96
A_15_P144886	NM_212439	period homolog 4	-4.52	-2.91
A_15_P144901	ENSDART00000045089		2.41	-
A_15_P145966	ENSDART00000051616	hypothetical LOC557008	-2.91	-2.52
A_15_P146096	ENSDART00000051505	similar to chemokine receptor D6	1.35	2.15
A_15_P146356	ENSDART00000098273	hypothetical LOC566714	2.27	-
A_15_P148046	NM_001100144	gastric inhibitory polypeptide	2.55	-
A_15_P148136	NM_001098257	insulin-like growth factor binding protein-1b	2.67	-
A_15_P149021	NM_001020787	interleukin 17a/f1	-	3.01
A_15_P150036	NM_200733	pyrophosphatase (inorganic)	-2.15	-2.16
A_15_P150101	NM_001020565	zgc:110064	2.19	-
A_15_P150431	NM_001076593	zgc:153394	-2.89	-2.10
A_15_P150521	NM_001037690	zgc:123280	-2.33	-2.37
A_15_P150526	NM_001025474	zgc:112368	3.00	-
A_15_P150751	NM_001080022	zgc:153767	-	-2.71
A_15_P151116	NM_001017909	zgc:112992	-2.88	-7.51
A_15_P151491	NM_173257	MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	-	-2.07
A_15_P151696	NM_001044973	si:dkey-94n12.3	2.16	-
A_15_P152006	NM_001007322	myosin binding protein C, slow type	-	2.56
A_15_P153321	NM_131253	opsin 1 (cone pigments), medium-wave-sensitive, 1	2.15	2.46
A_15_P153511	NM_001080059	glucose-dependent insulinotropic polypeptide	2.31	-
A_15_P153641	NM_200914	abhydrolase domain containing 2	-2.14	-2.06

A_15_P155136	ENSDART00000043226		-2.86	-1.79
A_15_P155226	ENSDART00000082465	solute carrier family 39 (zinc transporter), member 4	-	-2.79
A_15_P155481	ENSDART00000097408	similar to LOC560949 protein	2.37	3.43
A_15_P156806	ENSDART00000081029	similar to MGC53544 protein	3.41	3.19
A_15_P157246	NM_001008579	zgc:92239	-	-2.16
A_15_P157521	ENSDART00000043936		2.03	-
A_15_P158259	ENSDART00000014683		-	2.56
A_15_P158856	NM_213421	APEX nuclease (multifunctional DNA repair enzyme) 1	-	-2.70
A_15_P159091	ENSDART00000102534	si:dkey-207j16.6	-	2.40
A_15_P159371	ENSDART00000067011	hypothetical LOC567234	-2.12	-
A_15_P160152	ENSDART00000008810	similar to Tumor differentially expressed 2	2.36	2.10
A_15_P160691	NM_001037101	PASG	-	-3.13
A_15_P160696	NM_001079996	zgc:154042	2.68	-
A_15_P161031	NM_001037428	zgc:123097	-	-2.58
A_15_P161566	NM_131786	cryptochrome 3	-4.39	-2.95
A_15_P161661	NM_001030266	zgc:114168	2.53	-
A_15_P162166	NM_001017864	zgc:110697	2.14	2.35
A_15_P163936	NM_001002405	arrestin 3, retinal (X-arrestin), like	-1.78	-2.46
A_15_P162951	NM_200272	zgc:56628	2.09	-
A_15_P164071	NM_001080631	zgc:158792	1.78	2.25
A_15_P164116	NM_001017746	zgc:112531	2.27	-
A_15_P164676	ENSDART00000035654	hypothetical LOC572114	2.15	-
A_15_P165256	ENSDART00000101463	hypothetical protein LOC100005979	-	2.14
A_15_P165726	ENSDART00000076749	SAM domain, SH3 domain and nuclear localisation signals, 1	-	2.39
A_15_P165946	ENSDART00000051966	similar to Clca1 protein	2.74	-
A_15_P166596	NM_131400	thyrotroph embryonic factor	-2.40	-2.52
A_15_P166631	ENSDART00000105802	similar to Wnt3	-2.03	-
A_15_P167021	ENSDART00000105508		-	-2.21
A_15_P167691	ENSDART00000092421		-	2.30
A_15_P167811	ENSDART00000100832		-	2.11
A_15_P167831	ENSDART00000060172	hypothetical LOC557339	2.15	-
A_15_P168016	ENSDART00000100131	si:ch211-242e8.1	-2.80	-1.76
A_15_P168101	NM_131790	cryptochrome 1b	-1.92	-2.39
A_15_P168471	ENSDART00000088309	similar to mixed lineage kinase 2	-	2.75
A_15_P169136	NM_001020536	zgc:109962	1.82	2.30
A_15_P169291	NM_001077719	zgc:153935	-2.48	-1.55
A_15_P169381	NM_131547	Rh50-like protein	-	-2.94
A_15_P169411	NM_001082819	RORgamma-A	3.28	2.72
A_15_P169496	NM_001098772	zgc:165502	-1.64	-2.56

A_15_P169621	zgc:152898	NM_001077722	2.01	-
A_15_P169776	NM_001030281	MEP1A protein	-	-2.35
A_15_P169966	NM_178436	MCM5 minichromosome maintenance deficient 5 (S. cerevisiae)	-	-2.16
A_15_P170086	NM_001089470	zgc:162183	-	2.01
A_15_P171336	NM_001045210	zgc:113032	-2.11	-2.12
A_15_P171766	NM_001080600	SHQ1 homolog (S. cerevisiae)	-	4.28
A_15_P172406	NM_131667	GTP cyclohydrolase 2	-2.83	-3.58
A_15_P172761	NM_001089408	similar to Acheron	4.35	4.00
A_15_P172846	NM_199970	non-metastatic cells 2-like, protein (NM23B) expressed in	-2.39	-1.94
A_15_P172886	NM_200559	zgc:66109	-1.69	-2.03
A_15_P173156	NM_001045400	zgc:153443	-2.83	-2.64
A_15_P173191	NM_131792	cryptochrome 2b	3.91	2.66
A_15_P173416	NM_001024446	alpha-2,3-sialyltransferase v2	2.29	3.00
A_15_P173426	NM_001045367	zgc:152970	-2.03	-
A_15_P173841	NM_001030152	si:ch211-240l19.5	2.22	-
A_15_P175166	NM_001080655	zgc:158748	-	2.53
A_15_P175721	NM_001007132	zgc:123068	-	3.25
A_15_P176381	NM_001083859	zgc:162331	-2.01	-
A_15_P177056	ENSDART00000043507	si:ch211-284a13.1	-7.07	-5.27
A_15_P177151	BC108026		-	2.19
A_15_P177256	BC115099		1.40	2.80
A_15_P178836	BC150339		-2.80	-
A_15_P179346	NM_001109737	zgc:171699	-	2.18
A_15_P180961	ENSDART00000101151	similar to Syncollin	2.11	-
A_15_P182441	ENSDART00000055619		-	3.79
A_15_P183391	ENSDART00000010270	similar to Major facilitator superfamily domain containing 4	2.49	-
A_15_P183481	ENSDART00000054685	hypothetical LOC563546	-4.26	-3.56
A_15_P183531	ENSDART00000047784	similar to QEK5 protein	-	2.26
A_15_P183756	ENSDART00000033429	similar to opioid receptor-like protein Zf-orl	-	2.55
A_15_P184026	NM_001110035	zgc:171755	-2.57	-2.04
A_15_P184201	ENSDART00000026234	meprin A, alpha.2	-	-2.10
A_15_P185221	NM_001007038	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	-2.06	-2.98
A_15_P185766	ENSDART00000087300	hypothetical LOC566922	-1.68	-2.22
A_15_P185801	ENSDART00000023686	hypothetical LOC569518	-3.66	-3.05
A_15_P185901	ENSDART00000103833	similar to Slc26a6 C	2.37	-
A_15_P187316	NM_205729	nuclear receptor subfamily 1, group d, member 1	-12.23	-11.13
A_15_P188231	ENSDART00000043204	hypothetical LOC563995	-	2.19
A_15_P188331	ENSDART00000074388	hypothetical LOC556193	-	-2.25
A_15_P188516	NM_200586	myoglobin	2.62	-

A_15_P188666	ENSDART00000040156	hypothetical protein LOC793064	2.05	1.81
A_15_P188671	ENSDART00000073694	similar to Smu-1 suppressor of mec-8 and unc-52 homolog (C. elegans)	-2.78	-3.88
A_15_P188946	ENSDART00000081657	hypothetical LOC560602	2.69	4.96
A_15_P190351	ENSDART00000106687	hypothetical protein LOC798372	-	2.05
A_15_P190371	ENSDART00000055728	similar to TBC1 domain family, member 2	-	2.18
A_15_P190421	ENSDART00000106671		-	3.26
A_15_P190576	ENSDART00000040383	hypothetical LOC556714	2.23	-
A_15_P191926	ENSDART00000013114		5.77	7.33
A_15_P192081	ENSDART00000104017	hypothetical protein LOC796555	-	3.00
A_15_P192721	NM_001077453	zgc:153647	-	-3.40
A_15_P193091	NM_001089401	zgc:162907	-2.36	
A_15_P193906	NM_001011660	guanylate cyclase activator 1g	-2.86	-3.25
A_15_P193916	NM_001031841	G-protein-coupled receptor kinase 7a	-2.81	-2.01
A_15_P194791	NM_001024386	integrin, beta 4	-2.13	-
A_15_P195056	NM_001044839	si:dkey-14d8.6	2.16	-
A_15_P196291	NM_001011661	guanylate cyclase activator 1d	-2.92	-2.46
A_15_P196411	NM_200692	N-myc downstream regulated gene 1, like	4.06	4.11
A_15_P196701	ENSDART00000065660	similar to nuclear receptor subfamily 0, group B, member 2	3.24	-
A_15_P197381	ENSDART00000037585	hypothetical LOC557043	2.67	-
A_15_P197441	ENSDART00000057600	hypothetical LOC568302	-	-2.21
A_15_P197486	ENSDART00000051886	similar to MGC82104 protein	-	-2.38
A_15_P197596	ENSDART00000078346	similar to SH2 domain containing 4B	2.10	-
A_15_P197856	ENSDART00000085319	similar to Son of sevenless homolog 2 (Drosophila)	-	2.03
A_15_P198476	NM_001031841	G-protein-coupled receptor kinase 7a	-2.04	-2.25
A_15_P198681	ENSDART00000052065	hypothetical LOC563472	-	-2.84
A_15_P198761	ENSDART00000087687	hypothetical LOC568099	1.78	2.18
A_15_P198866	ENSDART00000057462	similar to MAM domain containing 4	-1.73	-2.17
A_15_P198956	ENSDART00000056577	similar to Retinol-binding protein I, cellular (Cellular retinol-binding protein) (CRBP)	-	3.09
A_15_P199141	ENSDART00000042974	similar to EAAT1	-2.31	-2.19
A_15_P201121	ENSDART00000062940	hypothetical protein LOC100002385	-	2.47
A_15_P201166	ENSDART00000062292	similar to activating transcription factor 2	-	4.23
A_15_P201661	ENSDART00000004619	hypothetical LOC558093	-2.19	-1.50
A_15_P201281	ENSDART00000011757	collagen, type IV, alpha 2	-	2.34
A_15_P201816	ENSDART00000077937	hypothetical LOC566497	-	-2.13
A_15_P202031	ENSDART00000085738		-	-2.29
A_15_P202046	ENSDART00000082368	similar to putative scavenger receptor MARCO	2.44	-
A_15_P202616	ENSDART00000104161		-	-3.61
A_15_P203161	ENSDART00000104161	similar to novel 7 transmembrane receptor (rhodopsin family)	7.44	4.37
A_15_P203856	ENSDART00000091183	hypothetical LOC565909	-	2.41

A_15_P203951	ENSDART00000080372	-3.43	-3.43	-3.43
A_15_P204061	ENSDART00000002751	similar to Sphingomyelin phosphodiesterase, acid-like 3B	-	-2.18
A_15_P204191	ENSDART00000009774	hypothetical protein LOC100006926	-	2.36
A_15_P204926	NM_200858	zgc:77748	-	-2.50
A_15_P204936	NM_131400	thyrotroph embryonic factor	-3.30	-2.11
A_15_P206011	NM_001045115	si:ch211-63o20.5	-2.32	-1.91
A_15_P206036	NM_001079657	zgc:158340	-2.99	-
A_15_P206101	NM_001045055	si:ch211-132b12.7	-4.85	-5.67
A_15_P207166	NM_001020557	zgc:110025	1.93	2.22
A_15_P207586	NM_001030267	zgc:114203	-	2.28
A_15_P208051	NM_001039890	si:dkey-3n7.3	4.72	4.36
A_15_P208126	NM_001004666	zgc:103645	-	3.72
A_15_P208246	NM_001077310	zgc:153102	3.03	-
A_15_P208311	NM_001003446	zgc:92530	2.58	-
A_15_P208526	NM_001076557	zgc:153679	-1.84	-2.60
A_15_P209096	ENSDART00000063389		3.01	-
A_15_P211836	NM_001030280	solute carrier family 24, member 5	-	2.50
A_15_P212516	BC124513	forkhead box P1a	-	2.61
A_15_P212631	BC134075		-6.97	-5.74
A_15_P213183	NM_001039624	odorant receptor	-	2.79
A_15_P224061	XM_684482		2.43	-
A_15_P215921	NM_001037385	solute carrier family 22 (organic cation transporter), member 18	-	2.43
A_15_P218651	TC348083		-	4.34
A_15_P219596	ENSDART00000101347	hypothetical protein LOC799808	-	2.16
A_15_P222321	ENSDART00000074896		-	2.76
A_15_P224021	TC316412		-	2.47
A_15_P226721	NM_001076593	zgc:153394	-2.74	-3.51
A_15_P227491	TC318046		-	2.02
A_15_P228111	NM_001006083	zgc:101667	-	-3.78
A_15_P228786	EH999121		-	2.33
A_15_P229176	EB935053		-2.86	-
A_15_P230491	EE205554		-2.07	-
A_15_P232521	EH502679		-	-2.12
A_15_P232661	EH511842		-	4.33
A_15_P234591	EH596069		-	2.12
A_15_P236266	NM_001030183	period homolog 1 (Drosophila)	-9.82	-6.41
A_15_P241411	AL925363		-	2.30
A_15_P242891	ENSDART00000082490	similar to K+ channel tetramerization protein	2.20	1.53
A_15_P243506	XM_694154	hypothetical LOC570649	3.60	3.91

A_15_P246021	DN901390		-	3.08
A_15_P246931	BC155059		-4.45	-2.65
A_15_P247446	EE686394		-	-2.26
A_15_P250981	CO351285		-	3.14
A_15_P251976	CV110355		-	3.06
A_15_P252806	DN599439		-	3.64
A_15_P254451	DT883475		-	2.56
A_15_P254726	DV598736		2.37	3.58
A_15_P257341	Y389459	toll-like receptor 21	2.03	3.66
A_15_P259081	XM_001338656	hypothetical protein LOC798239	-	2.80
A_15_P259686	XM_001339096	hypothetical protein LOC100004871	-	2.10
A_15_P259981	NM_001005946	zgc:103681	-	-2.55
A_15_P260781	NM_001003446	zgc:92530	3.73	-
A_15_P262061	AI444433	wu:fb38d07	-	3.13
A_15_P262786	CF595273	ribosomal protein S7	3.22	4.20
A_15_P263281	CN168062		-1.79	-2.28
A_15_P263411	BC096780	cryptochrome 2b	3.30	4.02
A_15_P263661	CT639065	wu:fc63c08	2.35	5.42
A_15_P266921	CN168062	wu:fj43c12	-	3.43
A_15_P269346	ENSDART00000106078		2.21	2.17
A_15_P269561	CT731659	hypothetical LOC567878	-	2.15
A_15_P271316	XM_001345452	similar to peptidylaminoacyl-L/D-isomerase	2.13	2.10
A_15_P271441	BC096780	interleukin 7 receptor	-	3.67
A_15_P272306	NM_001110021	carboxypeptidase B1 (tissue)	3.55	-
A_15_P274886	TC315630		2.87	-
A_15_P275176	CN168062		-	2.32
A_15_P275181	CN168062	bridging integrator 2	-	-2.59
A_15_P275781	TC316122		3.35	4.77
A_15_P278446	XM_689666	hypothetical LOC566399	-	-2.25
A_15_P278841	TC343394		-	2.08
A_15_P280791	TC344055		-	2.80
A_15_P280961	TC344117		-3.94	-2.80
A_15_P283191	NM_001037428	zgc:123097	-	-2.73
A_15_P284626	ENSDART00000102525		-2.72	-
A_15_P285436	ENSDART00000099439	hypothetical protein LOC100001311	-	3.96
A_15_P288956	XM_001344560	hypothetical protein LOC100005588	2.21	2.59
A_15_P289391	CK396210	hypothetical LOC556736	-	2.22
A_15_P290051	XM_001334265	similar to zinc finger of the cerebellum 1	-	5.98
A_15_P290916	XM_001332002	similar to Wu:fk63e10 protein	4.91	3.38

A_15_P292696	TC363916		-	2.79
A_15_P295001	TC364538		-	2.89
A_15_P295031	TC364547		-	3.26
A_15_P297976	BC092811		2.46	-
A_15_P298126	NM_001040251	zgc:136872	2.68	-
A_15_P298356	XM_682578	hypothetical LOC559258	-	2.35
A_15_P300356	EB942130		-	2.51
A_15_P302861	TC352260		-	2.14
A_15_P303381	TC352423		-	3.09
A_15_P303431	TC352442		-	2.72
A_15_P303531	XM_001335593	hypothetical protein LOC799059	-2.30	-1.49
A_15_P304011	TC352635		-	2.32
A_15_P305511	TC353105		-	2.48
A_15_P305641	CT591646		-	2.47
A_15_P305731	TC353185		-	4.88
A_15_P308261	NM_001099240	zgc:154142	-	-2.45
A_15_P308396	ENSDART00000103833	similar to Slc26a6 C	2.20	-
A_15_P308831	CD283185		-	2.40
A_15_P309601	XM_001342053	hypothetical protein LOC100002262	2.12	-
A_15_P309716	EE690478		2.91	-
A_15_P311056	EE203571		-	2.04
A_15_P313936	CT650977		-	2.03
A_15_P317051	EB830191		-	2.32
A_15_P318121	EH531036		-	3.10
A_15_P318196	EE204985		-	2.04
A_15_P318581	CT725582		-	2.83
A_15_P318841	CT736991		-	2.12
A_15_P319046	TC344353		-	2.36
A_15_P319486	TC344505		-	2.63
A_15_P321731	TC345231		-	5.19
A_15_P322763	TC345590		-	2.42
A_15_P324316	TC346137		-	3.76
A_15_P325286	CT692152		-	3.98
A_15_P325956	CT733358		2.00	3.07
A_15_P326496	CT688180		-	2.13
A_15_P329931	CT708607		-2.09	-
A_15_P331459	TC360823		-	5.73
A_15_P332779	TC361229		-	-2.15
A_15_P333534	TC361458		2.25	2.67
A_15_P334824	TC361867		-	2.03

A_15_P335194	TC361981		-	-2.35
A_15_P335504	TC362099		-	2.34
A_15_P336640	XM_693742	SET domain containing 5	-	2.30
A_15_P338720	TC363080		-	3.82
A_15_P339265	TC363245		2.07	1.60
A_15_P339845	TC363420		1.70	2.03
A_15_P347220	TC333289		-	2.71
A_15_P350505	TC334544		-	2.38
A_15_P351415	CT631401		-	2.83
A_15_P353140	CT731542		-	2.45
A_15_P353355	CT688213		-	2.38
A_15_P353640	CT623166		-2.36	-
A_15_P354090	CT673684		-	2.51
A_15_P354190	CT702703		-	2.60
A_15_P358140	CT686876		-3.99	-
A_15_P358445	CT695905		-	3.45
A_15_P359680	CN168295		-6.73	-4.00
A_15_P359710	CT672419		-	2.56
A_15_P360105	CT706569		-	2.55
A_15_P361565	CT711464		-	2.85
A_15_P363340	ENSDART00000097915		6.59	-
A_15_P364405	NM_001039890	si:dkey-3n7.3	2.10	2.41
A_15_P365520	NM_001020536	zgc:109962	1.78	2.28
A_15_P367020	NM_001033732	zgc:114126	-	2.01
A_15_P367705	TC308620		-2.38	-1.82
A_15_P368095	BC155350		-	-3.00
A_15_P368790	CT606464		-	2.59
A_15_P369485	CT736611		-	2.22
A_15_P369710	EH998102	wu:fc32g11	-	2.01
A_15_P369935	XM_001344193	hypothetical protein LOC100005092	1.83	2.05
A_15_P371510	EE689330	im:6912096	-	3.26
A_15_P372385	CT730275		-	2.16
A_15_P372920	EE329293		1.48	2.21
A_15_P373125	BI866341	wu:fb72d11	-3.13	-
A_15_P374010	EB966777		-	2.19
A_15_P374990	EH450222	hypothetical LOC557975	-2.29	-
A_15_P374230	EH583758		-	2.86
A_15_P374540	AL924095		-	2.33
A_15_P376390	NM_001089401	zgc:162907	-2.27	-2.14
A_15_P376945	XM_691260	hypothetical LOC567946	-	3.07

A_15_P378720	XM_001335658	similar to Gamma crystallin B (Gamma crystallin II)	-	2.51
A_15_P378930	XM_001335565	hypothetical protein LOC795423	-	2.97
A_15_P379375	TC355423		-	4.94
A_15_P381345	TC356055		-	2.22
A_15_P382570	TC356482		-2.11	-
A_15_P386305	CT614185		-	2.86
A_15_P386790	CT586615		-	2.16
A_15_P386810	CT601103		-	2.28
A_15_P388395	CT588602		-	2.04
A_15_P388405	CT588636		-	2.46
A_15_P389025	A_15_P389025		-	2.30
A_15_P389365	CT618214		-	2.03
A_15_P391640	EH475578	wu:fj39g12	-	2.02
A_15_P392370	CT674856		-	3.38
A_15_P394265	XM_680029	similar to novel 7 transmembrane receptor (rhodopsin family)	4.75	5.64
A_15_P394380	EL651135		4.54	4.00
A_15_P394650	CK024843		-	2.30
A_15_P399615	NM_001110481	zgc:162150	-1.98	-2.30
A_15_P401915	CR927166	hypothetical LOC562717	1.67	2.21
A_15_P403580	AW232471		-2.17	-
A_15_P405850	BI980905		-	2.02
A_15_P407880	CK360637	wu:fc23d08	-	4.18
A_15_P408365	NM_200371	zgc:64051	2.23	
A_15_P411150	ENSDART00000091905		-	3.84
A_15_P413080	NM_001077722	zgc:152898	2.02	-
A_15_P413960	TC328927		2.21	3.31
A_15_P415460	ENSDART00000008810	similar to Tumor differentially expressed 2	-	2.66
A_15_P415750	TC329665		-	4.05
A_15_P416845	TC330079		-2.82	-1.65
A_15_P417665	TC330397		-	2.28
A_15_P419550	NM_131667	GTP cyclohydrolase 2	-	-8.06
A_15_P419620	TC341009		-	2.15
A_15_P419955	NM_213178	regulator of chromosome condensation 1	-	3.29
A_15_P420430	TC341274		-	3.05
A_15_P420960	NM_001037690	zgc:123280	-2.59	-3.50
A_15_P421305	NM_200825	zgc:73075	-1.90	-3.37
A_15_P422020	TC341819		2.11	3.00
A_15_P422690	TC341819		-	2.03
A_15_P423620	TC342050		-	4.79
A_15_P423795	TC342429		-2.07	-

A_15_P423805	TC342431		-	2.22
A_15_P428200	CT605017		-	3.58
A_15_P429050	CT628419		-	2.43
A_15_P430240	EB855957		-1.57	-2.07
A_15_P430680	A_15_P430680		-	3.00
A_15_P433315	TC331696		-	2.26
A_15_P433820	BC131854	basic helix-loop-helix domain containing, class B, 3 like	-3.60	-2.72
A_15_P435710	TC332639		-	2.61
A_15_P437940	TC350648		-	2.63
A_15_P438615	TC350858		-	2.65
A_15_P439000	TC350973		-	2.24
A_15_P439415	XM_693448	similar to Rho GTPase activating protein 24	-	-2.08
A_15_P441135	ENSDART00000053878		-	3.98
A_15_P445490	NM_001045248	zgc:136342	-2.05	-
A_15_P447010	NM_001012485	bloody fingers	2.38	-2.06
A_15_P447820			-	2.13
A_15_P448870	EH565417		-2.05	-
A_15_P449805	AI943111		-	2.78
A_15_P451195	BG728808		-	2.33
A_15_P452670	EH438046		1.62	2.39
A_15_P453500	AW420829		1.73	2.24
A_15_P454180	BG305747		-	2.14
A_15_P455140	BI878623		-	4.75
A_15_P458000	BI475899		-	2.27
A_15_P459290	EE703225		-	2.95
A_15_P459940	CT708543		-	3.01
A_15_P460410	CO812634		-	3.46
A_15_P464200	AI878648		-	2.36
A_15_P465255	AW165029		-2.12	-
A_15_P467610	TC354275		1.58	2.14
A_15_P469570	TC354906		-	2.02
A_15_P469650	TC354933		-2.20	-1.64
A_15_P470115	TC355081		2.00	3.88
A_15_P471925	TC334983		-	3.36
A_15_P472415	TC335154		1.72	2.97
A_15_P472645	XM_684040	similar to glycerophosphodiester phosphodiesterase 2	-	2.05
A_15_P472765	TC335278		3.10	2.00
A_15_P473010	TC335357		-	4.00
A_15_P474625	TC335954		-	-3.27

A_15_P476655	CT649594		-	4.01
A_15_P476910	CT653988		-	2.36
A_15_P476960	CT623784		-	2.41
A_15_P477900	CT636524		-	2.81
A_15_P478835	CT731857		-	4.79
A_15_P479260	CT666198		2.02	3.08
A_15_P481125	CT681073		-	2.48
A_15_P481925	CT596461		-	2.47
A_15_P482005	CT637568		-	2.22
A_15_P482500	CT612049		-	4.10
A_15_P484195	TC348669		-	3.06
A_15_P484345	TC348705		-	-2.53
A_15_P485460	TC349070		-	3.58
A_15_P485495	TC349078		-	2.17
A_15_P487485	TC349720		-2.01	-2.31
A_15_P488585	TC349720	hypothetical protein LOC100000824	-	3.02
A_15_P489657	EE690052	hypothetical protein LOC796736	-	2.31
A_15_P491697	AL926171		-	-4.12
A_15_P493442	ENSDART00000085738	zgc:153269	-	-3.58
A_15_P495077	NM_001077548	zgc:154054	-	-2.76
A_15_P500277	NM_001077724	hypothetical protein LOC100001741	-	2.13
A_15_P500527	XM_001341647	hypothetical protein LOC791562	-	2.29
A_15_P500942	EH434502	similar to Slc5a8 protein	-	2.62
A_15_P501492	BM776399	hypothetical LOC561397	2.81	2.72
A_15_P502402	NM_213379	glutamic-oxaloacetic transaminase 2a, mitochondrial (aspartate aminotransferase 2)	-	-2.09
A_15_P503747	TC321541		-	2.06
A_15_P505562	TC322396		-	2.26
A_15_P505732	TC322491		-	2.30
A_15_P506732	TC338714		-	5.11
A_15_P509242	CT652640		-2.08	-1.93
A_15_P509602	CT670730		-	-3.99
A_15_P509712	TC339744		-	8.11
A_15_P510292	TC339972		-	3.24
A_15_P510937	TC340210		3.28	-
A_15_P511872	TC340549		-	2.05
A_15_P512177	TC308992		-	3.22
A_15_P513927	TC310020		-	2.23
A_15_P514182	TC310162		-	2.59

A_15_P517652	XM_001342326	similar to Tceb3 protein	-	2.54
A_15_P518137	NM_001113336	chromosome 6 open reading frame 85-like	1.31	2.09
A_15_P519787	CT670730		-	-2.49
A_15_P522977	CT716541		-	2.84
A_15_P523502	CT649297		-	2.46
A_15_P524847	CT680479		-	2.57
A_15_P525547	CT695215		3.88	4.51
A_15_P526882	CT638051		-2.45	-
A_15_P527677	CT626710		-	2.15
A_15_P528372	ENSDART00000099926	si:ch211-219a15.4	2.77	2.10
A_15_P529657	BC058069	similar to nuclear receptor subfamily 0, group B, member 2	3.39	-
A_15_P531517	TC326163		-	3.28
A_15_P532052	TC326380		-3.68	-
A_15_P532382	NM_001077625	cytokine receptor family member b7	4.48	-
A_15_P533222	TC359125		-	2.17
A_15_P534387	TC359501		2.59	2.50
A_15_P534632	TC359578		2.28	
A_15_P535392	TC359819		1.62	2.01
A_15_P535487	TC359853		2.00	3.82
A_15_P538302	NM_001030152	si:ch211-240l19.5	2.19	-
A_15_P539017	TC365088		-	3.02
A_15_P539682	TC365267		-	2.67
A_15_P541482	TC323411		-	2.18
A_15_P543477	TC324222		-3.29	-
A_15_P544897	NM_182891	opsin 1 (cone pigments), medium-wave-sensitive, 2	2.61	2.16
A_15_P546127	EH492283	similar to hCG1811317	-	-2.26
A_15_P546287	NM_131547	Rh50-like protein	-	-2.68
A_15_P546292	XM_687194	similar to dilute suppressor	5.17	7.15
A_15_P546422	ENSDART00000056274	hypothetical protein LOC793909	-	2.15
A_15_P547707	XM_001340852	similar to laminin alpha 1	-	5.67
A_15_P548947	XM_001336219	similar to Pnpo protein	-	4.53
A_15_P549472	XM_001340512	hypothetical protein LOC100002731	2.03	1.90
A_15_P551682	AL921834		2.04	4.13
A_15_P551817	XM_001338394	hypothetical protein LOC797982	2.03	-
A_15_P557897	DV585246		-	3.26
A_15_P557947	CT733242		-	2.08
A_15_P557992	CT603811		-	7.37
A_15_P558422	XM_001337807	hypothetical protein LOC797371	-	-2.21
A_15_P558612	EH514414		-	2.44

A_15_P558852	EH522417		1.92	2.18
A_15_P559492	EH540378		-	2.39
A_15_P559622	EH543423		2.02	-
A_15_P561042	EH471363	hypothetical LOC560023	-	2.27
A_15_P561347	EH459661		-	3.56
A_15_P561512	TC336646		1.89	2.42
A_15_P564302	TC337698		-	2.30
A_15_P564457	TC337764		-	-2.43
A_15_P564722	NM_001080652	zgc:158791	-	2.68
A_15_P565687	TC338188		-	-2.36
A_15_P567422	NM_001089476	zgc:162225	-	2.09
A_15_P567677	XM_001339077	hypothetical protein LOC798678	-	2.27
A_15_P567697	XM_001345243	hypothetical protein LOC100006575	1.75	2.45
A_15_P568002	XM_001344538	similar to LOC560949 protein	2.01	2.29
A_15_P569042	NM_001099993	hypothetical LOC558964	-	-2.04
A_15_P569632	CT680561		-	2.19
A_15_P570472	XM_683484	hypothetical LOC560089	1.86	2.27
A_15_P573132	ENSDART00000085185		-2.35	-
A_15_P573622	ENSDART00000013709		-3.12	-
A_15_P573797	EH459390		-	2.55
A_15_P574287	EL651166		-	2.14
A_15_P576122	CD759910		-	2.29
A_15_P576582	EE325777		-	-2.26
A_15_P577387	CR927034		-	2.92
A_15_P578012	CT681880		-	5.07
A_15_P580312	TC319482		3.89	4.91
A_15_P582117	TC320334		-	2.19
A_15_P582887	CN840802		-	2.03
A_15_P584927	AL914742		-	2.80
A_15_P585497	CD604692		-	2.35
A_15_P585942	EH464326		-	2.48
A_15_P586112	CD606517		-	2.59
A_15_P586902	CK142812		-	4.58
A_15_P587002	CK238860		-	2.05
A_15_P589387	EB940944		-	2.08
A_15_P589742	EE206580		-	2.82
A_15_P591802	AL729664	wu:fc31e09	-2.03	-1.53
A_15_P593992	ENSDART00000067200	hypothetical LOC560669	-3.16	-
A_15_P596397	ENSDART00000045089		2.42	-
A_15_P597092	AA658655	wu:fa55d03	-	2.22

A_15_P597232	EH995454		-	3.20
A_15_P597382	CV488726	wu:fa92c06	-	2.16
A_15_P597722	CT652640		-2.17	-1.93
A_15_P597972	XM_689666	hypothetical LOC566399	-	-2.51
A_15_P598077	AL928045		-	4.47
A_15_P599852	TC357259		-	2.57
A_15_P600772	TC357532		-	3.06
A_15_P603812	XM_686774	Relaxin 3c	-2.13	-
A_15_P603827	TC358489		-	2.16
A_15_P605152	TC358895		-	5.03

Table 2

100 top GO processes represented in MetaCore (FDR< 0.05)

Process	p-value
phototransduction	2.128e-09
detection of light stimulus	6.058e-09
positive regulation of cholesterol esterification	2.377e-08
regulation of cholesterol esterification	4.045e-08
detection of abiotic stimulus	4.897e-08
response to external stimulus	8.596e-08
digestion	1.803e-07
positive regulation of cGMP metabolic process	5.646e-07
positive regulation of cGMP biosynthetic process	5.646e-07
positive regulation of steroid metabolic process	9.739e-07
detection of external stimulus	1.133e-06
very-low-density lipoprotein particle remodeling	1.241e-06
regulation of cGMP biosynthetic process	1.251e-06
regulation of cGMP metabolic process	1.587e-06
cGMP-mediated signaling	1.587e-06
alcohol metabolic process	1.655e-06
lipid metabolic process	1.763e-06
regulation of steroid metabolic process	1.805e-06
cellular response to stimulus	1.843e-06
sensory perception of light stimulus	2.514e-06
visual perception	2.514e-06
positive regulation of low-density lipoprotein receptor catabolic process	2.792e-06
triglyceride-rich lipoprotein particle remodeling	2.885e-06

axon regeneration in the peripheral nervous system	2.885e-06
macromolecular complex remodeling	3.037e-06
plasma lipoprotein particle remodeling	3.037e-06
protein-lipid complex remodeling	3.037e-06
cholesterol catabolic process	4.139e-06
sterol catabolic process	4.139e-06
response to light stimulus	5.353e-06
phospholipid efflux	5.754e-06
multicellular organismal lipid catabolic process	6.933e-06
nitric oxide mediated signal transduction	7.565e-06
low-density lipoprotein particle remodeling	7.791e-06
negative regulation of cell migration	8.680e-06
plasma lipoprotein particle assembly	1.032e-05
protein-lipid complex assembly	1.032e-05
high-density lipoprotein particle remodeling	1.032e-05
steroid catabolic process	1.042e-05
cholesterol metabolic process	1.090e-05
regulation of membrane protein ectodomain proteolysis	1.340e-05
positive regulation of nitric-oxide synthase activity	1.340e-05
negative regulation of blood vessel endothelial cell migration	1.340e-05
regulation of cholesterol biosynthetic process	1.340e-05
negative regulation of cholesterol biosynthetic process	1.377e-05
regulation of low-density lipoprotein receptor catabolic process	1.377e-05
very-low-density lipoprotein particle clearance	1.377e-05
chylomicron remodeling	1.377e-05
regulation of fatty acid biosynthetic process	1.404e-05
negative regulation of cellular component movement	1.493e-05
regulation of lipid biosynthetic process	1.493e-05
negative regulation of locomotion	1.856e-05
sterol metabolic process	1.976e-05
regulation of lipid transport	2.076e-05
negative regulation of steroid biosynthetic process	2.152e-05
triglyceride metabolic process	2.274e-05
Cdc42 protein signal transduction	2.395e-05
positive regulation of interleukin-5 production	2.395e-05
positive regulation of interleukin-13 production	2.395e-05
negative regulation of platelet activation	2.395e-05
negative regulation of steroid metabolic process	2.671e-05
folic acid and derivative metabolic process	2.671e-05

reverse cholesterol transport	2.671e-05
regulation of MAP kinase activity	3.049e-05
positive regulation of nucleotide metabolic process	3.105e-05
positive regulation of cyclic nucleotide biosynthetic process	3.105e-05
positive regulation of nucleotide biosynthetic process	3.105e-05
positive regulation of cyclic nucleotide metabolic process	3.105e-05
steroid metabolic process	3.427e-05
response to radiation	3.449e-05
phospholipid transport	3.499e-05
entrainment of circadian clock	3.806e-05
chylomicron remnant clearance	3.806e-05
high-density lipoprotein particle assembly	3.806e-05
response to organic substance	3.852e-05
phototransduction, visible light	3.976e-05
response to dietary excess	3.976e-05
positive regulation of monooxygenase activity	3.976e-05
glycerolipid metabolic process	4.124e-05
cholesterol transport	4.918e-05
cellular lipid metabolic process	5.124e-05
sterol transport	5.478e-05
high-density lipoprotein particle clearance	5.672e-05
positive regulation of triglyceride catabolic process	5.672e-05
lipoprotein catabolic process	5.672e-05
positive regulation of fatty acid biosynthetic process	5.696e-05
positive regulation of lipid transport	5.696e-05
negative regulation of endothelial cell migration	5.696e-05
cholesterol efflux	5.696e-05
cellular aromatic compound metabolic process	5.841e-05
acylglycerol metabolic process	6.071e-05
vasodilation	6.087e-05
negative regulation of cellular protein metabolic process	6.328e-05
regulation of lipid metabolic process	6.585e-05
regulation of nitric-oxide synthase activity	6.734e-05
neutral lipid metabolic process	7.029e-05
regulation of circadian rhythm	7.458e-05
glycerol ether metabolic process	7.551e-05
regulation of lipoprotein lipase activity	7.903e-05
Spemann organizer formation at the anterior end of the primitive streak	7.954e-05

Table 3

Relevant maps and pathways represented in MetaCore (FDR< 0.05). Gray shapes mark maps for which additional experiments were conducted.

Map	Pathway	p-Value
Lipid Biosynthesis and regulation	- Regulation of lipid metabolism_RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR - Regulation of lipid metabolism_Insulin signaling:generic cascades	1.882e-3
Cholesterol and bile acid homeostasis	- Niacin-HDL metabolism - Transport_Intracellular cholesterol transport in norm - Cholesterol Biosynthesis	4.132e-3
Vascular development (Angiogenesis)	- Transcription_Role of Akt in hypoxia induced HIF1 activation - Transcription_Receptor-mediated HIF regulation - Development_FGFR signaling pathway	3.414e-2
Vitamin and cofactor metabolism and its regulation	- Niacin-HDL metabolism - Folic acid metabolism	3.421e-2
Mitogenic signaling	- Development_IGF-1 receptor signalling - Development_EGFR signaling via PIP3 - Development_FGFR signaling pathway - Development_EGFR signaling pathway - Development_Membrane-bound ESR1: interaction with growth factors signalling - Signal transduction_PTEN pathway - Development_HGF signaling pathway	4.713e-2
Apoptosis	- Transcription_P53 signaling pathway - Development_Flt3 signaling - Apoptosis and survival_Role of CDK5 in neuronal death and survival - Apoptosis and survival_BAD phosphorylation - Apoptosis and survival_Anti-apoptotic action of Gastrin - Apoptosis and survival_HTR1A signalling	5.423e-2
Nuclear receptor signaling	- Regulation of lipid metabolism_RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR - Development_Ligand-independent activation of ESR1 and ESR2 - Transcription_Androgen Receptor nuclear signalling - Transcription_PPAR Pathway	7.081e-2
Hematopoiesis	- Development_Flt3 signaling - Development_Thrombopoietin-regulated cell processes	9.665e-2
Estrogen signaling	- Development_Membrane-bound ESR1: interaction with growth factors signalling - Development_Ligand-independent activation of ESR1 and ESR2	1.094e-1

DNA-damage response	- Transcription_P53 signaling pathway - DNA damage_Role of Brca1 and Brca2 in DNA repair - DNA damage_Brca1 as a transcription regulator - DNA damage_Nucleotide excision repair - Apoptosis and survival_BAD phosphorylation	1.146e-1
Tissue remodeling and wound repair	- Development_EGFR signaling pathway - Development_EGFR signaling via PIP3 - Development_HGF signaling pathway - Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodelling	1.183e-1
Neurotransmission	- Development_Dopamine D2 receptor transactivation of EGFR - Neurophysiological process_NMDA-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons	1.266e-1
Immune system response	- Immune response_BCR pathway - Immune response_IFN gamma signaling pathway - Immune response_Regulation of T cell function by CTLA-4 - Immune response_IL-4 signaling pathway - Immune response_IL-2 activation and signaling pathway - Immune response_IL-15 signaling - Immune response_CD40 signaling	1.380e-1
Protein synthesis	- Translation _Regulation activity of EIF2 - Translation_Non-genomic (rapid) action of Androgen Receptor - Translation_Insulin regulation of translation - Translation _Regulation activity of EIF4F	1.599e-1
Androgen signaling	- Translation_Non-genomic (rapid) action of Androgen Receptor - Transcription_Androgen Receptor nuclear signalling	1.696e-1
Inflammatory response	- Immune response_Gastrin in inflammatory response - Immune response_CCR5 signaling in macrophages and T lymphocytes	1.860e-1
Energy metabolism and its regulation	- Regulation of lipid metabolism_RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR - Regulation of lipid metabolism_Insulin regulation of glycogen metabolism - Regulation of lipid metabolism_Insulin regulation of fatty acid methabolism	1.982e-1
Myogenesis regulation	- Development_EDG5 and EDG3 in cell proliferation and differentiation - Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	2.366e-1

Nucleotide metabolism and its regulation	- CTP/UTP metabolism	3.040e-1
Cell cycle and its regulation	- Cell cycle_Start of DNA replication in early S phase - Apoptosis and survival_nAChR in apoptosis inhibition and cell cycle progression - Cell cycle_Role of Nek in cell cycle regulation - Cell cycle_Transition and termination of DNA replication	3.339e-1
Vasodilation	- Development_A2A receptor signaling	3.614e-1
Calcium signaling	- Transcription_CREB pathway - Signal transduction_IP3 signaling - Signal transduction_PKA signaling	3.886e-1
Cardiac Hypertrophy	- Development_PIP3 signaling in cardiac myocytes	4.066e-1
Oxidative stress regulation	- Development_Dopamine D2 receptor transactivation of EGFR	4.140e-1
Cell differentiation	- Development_Regulation of epithelial-to-mesenchymal transition (EMT) - Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK. - Development_Beta-adrenergic receptors regulation of ERK	4.175e-1
Cystic fibrosis disease	- Cholesterol and Sphingolipids transport / Recycling to plasma membrane in lung (normal and CF)	5.200e-1
Vasoconstriction	- Cytoskeleton remodeling_Alpha-1A adrenergic receptor-dependent inhibition of PI3K - Immune response_HTR2A-induced activation of cPLA2 - Transport_Alpha-2 adrenergic receptor regulation of ion channels - Development_EDNRB signaling - Muscle contraction_ACM regulation of smooth muscle contraction	5.792e-1
Aminoacid metabolism and its regulation	- Histidine-glutamate-glutamine metabolism	5.928e-1

Appendix 3

Table 1

Significantly altered genes in zebrafish adult male after exposure to 2.2 µg/L and 890 µg/L EHMC (GeneSpring GX 11 adjusted p<0.05 and adjusted Fold Change≥2).

Agilent ID	Gene Symbol	GenBank Accession	Regulation	Fold change (log_2)	
				3 µg/L	3000 µg/L
A_15_P201331	ankzf1	BC090778	down	4.25	4.27
A_15_P187481	LOC572483	XM_001922419	up	2.31	2.35
A_15_P118515	ptgds	NM_213634	up	3.36	5.83
A_15_P131446		L48874	down	3.53	2.00
A_15_P178126	top2b	NM_001045191	down	2.62	4.13
A_15_P426955		CT713922	down	2.23	2.80
A_15_P224196			up	3.82	2.93
A_15_P398495	pcsk5b	NM_001083829	down	2.41	3.01
A_15_P103847	zgc:136383	NM_001045294	down	7.55	5.81
A_15_P349790	si:dkey-228a15.3	NM_001128533	down	3.36	3.29
A_15_P515257			down	35.79	7.96
A_15_P180906			up	5.21	3.02
A_15_P110272	LOC100149222	BC151959	down	2.63	5.10
A_15_P199381	LOC793259	XM_001332950	down	2.77	3.47
A_15_P325751		CT706689	down	2.83	3.01
A_15_P170916	arhgef11	NM_001031840	down	2.19	2.69
A_15_P161071	zgc:153253	NM_001160823	up	2.04	2.07
A_15_P118059	zgc:101566	NM_001005996	up	5.27	3.56
A_15_P309431		DT059977	up	2.96	3.39
A_15_P109473	gdf6a	NM_001159994	up	2.31	2.24
A_15_P159781	CH211-209N20.1	XM_001920619	down	2.80	17.33
A_15_P106118	zgc:110222	NM_001017643	up	2.00	2.52
A_15_P212111		BC076429	down	2.05	3.30
A_15_P106147		CO354205	down	10.52	6.67
A_15_P168216	si:dkey-261h15.1	NM_001080159	down	3.40	2.85
A_15_P115758	si:ch211-240l19.8	NM_001082930	up	2.33	2.47
A_15_P535007			down	3.72	2.32
A_15_P600377			down	2.97	2.25
A_15_P225051			down	13.17	25.10
A_15_P184996	dsc2l	BC124676	down	2.45	2.40
A_15_P179486	LOC566408	BC151988	down	3.23	2.83
A_15_P170386	si:dkey-4c15.14	NM_001089575	up	2.74	2.40
A_15_P133416	dnmt1	BC044335	down	3.36	4.37
A_15_P120419	rbp2a	AF363957	down	33.75	12.87
A_15_P109079	ddefl1	NM_001039992	down	2.39	2.32
A_15_P160886	rpa2	NM_131711	up	2.38	2.19
A_15_P472850			up	19.92	8.22
A_15_P101803	zgc:172075	NM_001114408	up	2.51	2.73
A_15_P584632		AL927245	up	4.06	8.47

A_15_P193121	edn1	NM_131519	up	2.49	2.37
A_15_P592857	wu:fj32b02	XM_683842	down	2.06	3.00
A_15_P197261		BC154509	up	6.83	5.03
A_15_P190501	thoc2	NM_001003847	down	2.22	4.25
A_15_P136451	atp2b1b	NM_001135631	down	2.35	2.72
A_15_P351055		CT647711	down	4.18	5.49
A_15_P243131	LOC100007935	XM_001346234	up	3.05	2.64
A_15_P100991	zgc:55398	NM_201048	up	2.72	2.35
A_15_P490392	LOC556882	XM_001919832	down	2.03	2.11
A_15_P102120	foxq1	NM_212907	up	3.08	3.03
A_15_P110501	tbcb	NM_212775	up	8.27	7.43
A_15_P552127	znf41	BC090732	down	2.99	3.00
A_15_P167706		XM_001921641	down	3.63	4.48
A_15_P173511	si:ch211-238n5.4	NM_001044347	up	2.71	2.49
A_15_P238716	top2b	NM_001045191	down	2.35	4.00
A_15_P225386			down	3.82	2.16
A_15_P441420			down	2.44	3.10
A_15_P155176	ggct	NM_213005	up	2.92	2.37
A_15_P429435	DKEY-45K15.2	NM_001144794	down	2.34	2.12
A_15_P120249	fgf13	NM_001007399	up	3.21	2.96
A_15_P456985		EB973958	up	2.46	2.70
A_15_P209466	slc35f2	NM_001076556	up	4.71	5.04
A_15_P390035		CT619466	down	6.64	3.91
A_15_P207781	gle1l	NM_001003885	down	2.15	2.37
A_15_P409440	zgc:162618	NM_001089331	up	2.72	7.93
A_15_P440410		XM_001336957	down	2.45	4.09
A_15_P407120	CH211-15B7.4	NM_001126472	up	3.45	4.89
A_15_P339525			down	3.26	6.45
A_15_P236606	mef2ca	NM_131312	down	3.43	3.98
A_15_P255401			down	3.66	2.59
A_15_P554702			down	3.45	3.12
A_15_P176881		BC096935	down	3.48	5.61
A_15_P100747	zgc:103434	NM_001006037	down	2.85	3.04
A_15_P229956		EB981970	up	8.42	3.59
A_15_P309736	f3a	BC154033	down	2.50	2.80
A_15_P150711	itgav	NM_001033721	up	2.85	2.21
A_15_P266611		CK015994	up	2.07	2.15
A_15_P184576	si:dkey-221h15.4	XM_687310	up	2.40	2.68
A_15_P397815		EH433662	up	6.23	4.95
A_15_P131516	hoxa13b	NM_131194	up	2.13	2.32
A_15_P105414	zgc:195155	NM_001128687	up	2.03	2.19
A_15_P101233	si:dkey-103i16.2	NM_001044974	up	4.34	3.47
A_15_P499802	LOC566173	BC152290	up	2.34	2.50

A_15_P416305	zgc:101867	NM_001008590	up	3.35	2.63
A_15_P144361	tppp3	NM_201335	up	2.28	2.32
A_15_P113187			up	2.16	2.12
A_15_P102389	wu:fb70b03	EE319212	up	8.13	9.01
A_15_P211851	vasn	NM_001018131	up	5.21	5.82
A_15_P287801	LOC558930	XM_682219	up	2.44	2.63
A_15_P107010	sult1st1	NM_182941	up	2.01	2.41
A_15_P186906		BC092801	down	2.94	2.54
A_15_P164881	znf41	BC155766	down	4.04	4.38
A_15_P441545	LOC566344	XM_689616	down	3.15	3.84
A_15_P171941	zgc:163047	NM_001089564	down	2.59	2.37
A_15_P241111	LOC797162	XM_001337584	down	3.07	4.36
A_15_P134556	ptenb	NM_001001822	down	2.51	3.07
A_15_P182186	nfe2l3	NM_213231	down	2.11	2.21
A_15_P227846			down	3.33	2.01
A_15_P223676			up	2.63	2.06
A_15_P288191			down	3.86	2.08
A_15_P278901			down	4.75	2.88
A_15_P179987	or137-5	NM_001130804	up	4.21	5.77
A_15_P144141			down	3.38	2.32
A_15_P423850		CT675248	down	3.19	2.15
A_15_P567152	akap1b	NM_001098179	down	2.23	2.64
A_15_P152216	vox	NM_131698	down	5.39	2.93
A_15_P159721			down	2.12	3.46
A_15_P305781			down	2.99	2.20
A_15_P105319			down	3.23	2.38
A_15_P272866			down	3.07	2.58
A_15_P104528	stm	NM_198817	up	2.86	2.83
A_15_P134231	xpc	NM_001045210	down	2.17	3.03
A_15_P582987		BQ262605	down	4.59	3.82
A_15_P535372			down	2.23	2.17
A_15_P355255		CT707445	down	11.08	12.52
A_15_P189811	si:ch211-218o21.2	NM_001123234	down	2.79	4.04
A_15_P199851	wu:fc18f06	XM_687839	up	5.17	5.45
A_15_P106063			down	3.04	2.20
A_15_P162001	rnd3b	NM_001002591	up	2.12	2.41
A_15_P154431	LOC100004902	BC115073	up	4.45	3.97
A_15_P144301	zgc:195027	NM_001130634	down	3.01	2.48
A_15_P443450	DKEY-221J11.2	XM_688722	up	3.62	3.49
A_15_P100039			up	4.02	4.33
A_15_P205056	ftr72	NM_001007387	down	2.95	3.07
A_15_P109375	LOC559157	XM_001919279	up	2.53	2.49
A_15_P181246			up	2.89	3.79

A_15_P399015			down	3.91	2.54
A_15_P239061			up	2.10	2.99
A_15_P410955			down	3.42	2.11
A_15_P130046		XM_690666	down	3.73	9.13
A_15_P203161		XM_680029	up	3.05	4.08
A_15_P400835	LOC556013	XM_678703	down	2.49	2.85
A_15_P365850	ptgs2b	NM_001025504	up	2.48	2.56
A_15_P181026		XM_685788	down	2.14	2.11
A_15_P346915			down	4.31	4.96
A_15_P100735	hoxb6b	NM_131538	up	3.57	3.48
A_15_P141281	LOC100002334	NM_001098260	up	3.25	3.12
A_15_P145796		BC100005	down	2.06	3.11
A_15_P120256	btg4	BC053242	up	2.26	3.36
A_15_P156841		EH585602	up	3.49	5.64
A_15_P217661			down	2.01	2.23
A_15_P176566	zgc:103736	NM_001013446	up	3.84	3.49
A_15_P104449	npy8br	NM_131436	down	3.16	3.09
A_15_P190706	cxcl14	NM_131627	up	3.08	2.91
A_15_P119543	rab3d	NM_001145597	down	3.06	2.17
A_15_P172886	zgc:66109	NM_200559	down	2.03	3.43
A_15_P484810		XM_687344	down	2.28	2.51
A_15_P597332		AI204746	down	3.02	2.16
A_15_P200391	setdb2	NM_207058	up	4.30	2.30
A_15_P141116	ctdsplb	NM_001076615	down	2.31	3.15
A_15_P568827		XM_685280	down	3.06	7.01
A_15_P230116	wu:fc18f06	EB992434	up	2.47	2.62
A_15_P135746	LOC571244	XM_001919024	up	3.50	5.71
A_15_P558047		EH547031	down	4.73	2.03
A_15_P147381	LOC560957	XM_684355	up	2.69	2.44
A_15_P255941		CT725570	down	2.75	2.52
A_15_P112432	nbas	NM_001044807	down	3.61	3.05
A_15_P121256	LOC798718	XM_001923006	down	2.82	2.55
A_15_P133521	zgc:66125	NM_200823	down	4.08	3.95
A_15_P368605	thbs1	XM_685303	up	2.03	2.53
A_15_P172876	lbx2	NM_001007134	up	3.98	2.92
A_15_P108326			down	2.58	2.22
A_15_P417790			down	4.95	5.03
A_15_P179011	hamp1	NM_205583	up	8.25	5.24
A_15_P190576	LOC556714	XM_679585	up	2.95	3.82
A_15_P134521	frap1	NM_001077211	down	2.99	3.91
A_15_P105833	znf143	BC124735	down	3.13	2.98
A_15_P283261			up	5.41	3.85
A_15_P130591	fgfr1a	NM_152962	down	3.02	4.62

A_15_P166066	LOC100150979	XR_044871	down	4.47	6.08
A_15_P309586	LOC573743	XM_001922667	down	2.30	2.25
A_15_P121123	mitfa	NM_130923	up	2.22	2.02
A_15_P134781	plp1a	NM_175576	down	2.58	3.69
A_15_P161296	sp8	NM_213241	up	4.17	3.68
A_15_P118368			down	4.20	2.22
A_15_P211753	LOC571647	NM_001109718	up	5.95	5.55
A_15_P178051		BC129436	down	2.74	2.09
A_15_P591527		CO925666	down	2.32	2.54
A_15_P310471	sgca	XM_680178	up	2.22	2.58
A_15_P568757			down	3.15	6.82
A_15_P410745	LOC565583	CO934890	up	2.37	2.54
A_15_P162446	usp22	NM_001045248	down	5.68	3.40
A_15_P448415		EH447279	down	3.35	6.73
A_15_P144841			down	3.15	5.85
A_15_P538917			down	4.67	6.38
A_15_P182231	si:dkey-151c10.1	NM_001100032	down	2.97	3.33
A_15_P514017			down	3.71	2.69
A_15_P103134	zgc:110540	NM_001014352	up	2.47	2.68
A_15_P208991	il17rd	NM_153660	up	2.45	2.55
A_15_P184876	si:ch211-57i17.5	XM_688399	up	4.45	2.76
A_15_P106551	nucks1	NM_001004601	down	2.44	4.24
A_15_P166541	im:7138263	AB247648	up	4.27	5.09
A_15_P378340	im:6894757	BC115086	up	2.59	2.41
A_15_P238121	skp2	NM_001082837	up	3.56	3.04
A_15_P207326	zgc:153637	NM_001077457	down	4.59	3.46
A_15_P119909	tln2	BC055166	down	2.33	2.81
A_15_P178271	rexo1	NM_001126416	down	2.17	3.24
A_15_P532767			up	3.80	3.77
A_15_P329226		CT665069	down	3.83	2.36
A_15_P103667	si:busm1-136d19.2	NM_001005312	up	2.82	4.12
A_15_P540367	bbs7	NM_001077145	down	2.40	2.40
A_15_P103392	LOC100148098	XM_001923612	up	3.16	2.86
A_15_P111238	nitr3a	NM_001007203	up	2.85	4.08
A_15_P100416	LOC100001774	BC076251	up	3.31	2.04
A_15_P152291	LOC561381	NM_001044958	up	10.68	6.74
A_15_P101461	si:ch211-240l19.7	NM_001082929	up	4.78	13.16
A_15_P208376	g12	NM_131335	up	2.36	2.46
A_15_P102830	hspb7	NM_001006040	up	2.36	2.24
A_15_P159361	LOC567778	XM_691079	up	2.62	2.86
A_15_P201486	si:ch211-219a4.6	BC129404	down	2.01	3.70
A_15_P116585		AL717593	down	3.78	4.75
A_15_P445490	usp22	NM_001045248	down	3.52	2.57

A_15_P102850	glra4a	NM_131782	up	3.75	4.23
A_15_P181131			up	3.22	3.35
A_15_P187676	LOC100001520	XM_001341462	up	4.86	3.09
A_15_P107437	synpr	NM_001004532	up	7.14	3.90
A_15_P224541			up	2.31	2.27
A_15_P275231	lrrc15	NM_001080682	up	3.33	2.67
A_15_P389740		CT701002	down	19.85	10.17
A_15_P565842	dhx37	NM_001083004	down	3.02	3.36
A_15_P134261	ttnb	BC090540	down	5.59	4.27
A_15_P227186			down	3.03	8.80
A_15_P421875			down	3.31	2.07
A_15_P216876			down	2.76	2.36
A_15_P178381		BC135030	down	2.49	4.84
A_15_P399615	zgc:162150	NM_001110481	up	4.03	4.81
A_15_P145596	ppm1f	AB113301	down	32.28	8.17
A_15_P210881	zgc:101848	NM_001005966	down	2.14	2.25
A_15_P364890	mybl2	NM_001003867	up	2.45	2.54
A_15_P395230	id:ibd2611	EE704940	up	3.15	2.69
A_15_P355430			down	4.09	2.34
A_15_P294351			down	10.82	6.90
A_15_P120601	nos2a	NM_001104937	up	3.24	3.08
A_15_P469530			up	9.08	7.46
A_15_P201191	itga6	NM_001144781	up	2.04	2.34
A_15_P190496	thoc2	NM_001003847	down	3.16	3.11
A_15_P491602	ercc1	NM_001103138	up	2.22	2.21
A_15_P106487	zgc:162523	NM_001089561	down	3.83	5.26
A_15_P178481	sart3	NM_001030118	down	2.74	3.51
A_15_P104409	pcna	NM_131404	up	2.06	2.18
A_15_P134026	uba52	BC076111	down	2.52	2.52
A_15_P115140		BM026700	down	3.64	2.76
A_15_P167751	ctcf	NM_001001844	down	2.04	4.91
A_15_P118928		XM_001340479	down	4.76	2.88
A_15_P321711			up	10.40	6.92
A_15_P362840	LOC100149042	XM_001923904	up	9.05	3.52
A_15_P101051	tchp	NM_001040343	down	2.37	2.76
A_15_P192406		BC090414	down	4.03	7.31
A_15_P117168		XM_692864	down	2.48	2.75
DCP_1_4			down	2.43	2.74
A_15_P119243	mafba	NM_131015	down	2.11	2.30
A_15_P173461	zgc:153239	NM_001075112	down	2.07	2.39
A_15_P382680			down	3.60	2.22
A_15_P390845			down	2.92	3.00
A_15_P162161	lmo2	NM_131111	up	2.49	2.10

A_15_P431930			down	2.97	2.91
A_15_P187641		BC115093	down	2.73	3.75
A_15_P331171	anxa1a	NM_181758	up	3.93	3.30
A_15_P582292	foxq1	NM_212907	up	2.98	2.76
A_15_P266951	zgc:110727	NM_214782	up	2.65	2.28
A_15_P184551	si:dkey-221h15.2	NM_001045025	up	3.50	2.75
A_15_P203477		BC154745	up	2.50	2.07
A_15_P110557	epn1	NM_213362	down	2.63	2.63
A_15_P153656	dgat2	NM_001030196	down	2.11	2.25
A_15_P446835			up	7.32	6.00
A_15_P138971			up	6.51	4.67
A_15_P133001	freqb	NM_001020514	up	2.37	2.05
A_15_P117390	LOC100007076	XM_001345596	down	3.18	4.76
A_15_P473640	zgc:158364	NM_001089409	up	4.09	4.80
A_15_P290831	sema4g	XM_001921388	down	2.00	4.55
A_15_P552892	zgc:174896	NM_001110412	up	2.85	2.14
A_15_P134366	taf2	NM_001003835	down	2.05	3.05
A_15_P256551	crb3b	NM_001045323	up	2.51	2.40
A_15_P158448	LOC100150616	XM_001921334	up	9.93	4.01
A_15_P571112	LOC557544	DT876649	up	5.27	5.33
A_15_P227811			down	17.25	6.25
A_15_P203731	zgc:66117	NM_199560	up	2.45	2.12
A_15_P120815		BC065887	up	7.59	6.96
A_15_P225991			up	5.13	4.01
A_15_P117301	LOC553499	BC077098	down	3.08	3.21
A_15_P158571	tln1	NM_001009560	down	2.57	2.16
A_15_P107284	si:dkey-69h6.7	NM_001100015	down	3.72	2.97
A_15_P188626	LOC100149222	BC151959	down	2.02	2.27
A_15_P481815		CT610756	down	4.18	2.14
A_15_P277851		XM_001343661	up	2.43	3.16
A_15_P190721	ppargc1b	XM_678566	down	2.05	2.40
A_15_P278586			up	2.43	2.88
A_15_P161656	itgb3b	NM_001082948	up	4.66	3.82
A_15_P131747	foxb1.1	NM_131283	up	2.08	3.30
A_15_P113732	LOC564883	XM_688207	up	3.40	3.89
A_15_P107408	acpl2	NM_001002430	down	2.63	4.75
A_15_P222111			down	3.95	3.36
A_15_P347895	top1l	NM_001044324	down	2.26	4.09
A_15_P590552	wu:fa56c05	AA658707	down	7.05	5.91
A_15_P108387	baz1a	BC081390	down	2.06	2.60
A_15_P119002	pcdh10a	NM_212571	up	2.03	2.38
A_15_P182446		BC134117	down	3.56	4.38
A_15_P245696		EH433136	down	5.18	2.62

A_15_P104390	sp8l	NM_205550	up	3.49	3.31
A_15_P542267	myom1a	NM_001161340	down	2.10	2.21
A_15_P463155		AI522379	down	2.34	3.96
A_15_P117331	srrm2	BC071371	down	2.68	3.87
A_15_P329166		CT708252	down	9.71	8.90
A_15_P293056			up	2.20	2.71
A_15_P578427	ccdc25	NM_200388	up	2.73	2.02
A_15_P120199		CA472755	up	2.18	2.36
A_15_P308321	LOC795147	XM_001335221	up	2.59	2.49
A_15_P101193	mxi1	NM_131237	down	3.12	4.10
A_15_P178601		BC142784	down	5.18	4.04
A_15_P339545			down	2.28	2.01
A_15_P160161	LOC571171	XM_694737	up	5.37	4.68
A_15_P498172	LOC100006752	BC154669	up	4.25	2.24
A_15_P550422	jmjd5	NM_001109869	up	3.32	2.28
A_15_P150471	defbl2	NM_001081554	up	4.01	3.19
A_15_P193756	fzd8b	NM_131553	up	2.53	2.18
A_15_P114909	zfand5b	NM_200949	up	4.64	2.19
A_15_P596927		XM_680992	down	2.26	2.13
A_15_P185551	lepr	NM_001113376	down	3.70	4.64
A_15_P271926			up	2.90	2.88
A_15_P102241	LOC100002224	XM_001922426	down	2.72	3.12
A_15_P120214		XM_001341599	down	3.40	4.29
A_15_P109915	aqp4	NM_001003749	down	2.30	4.67
A_15_P137333			down	2.99	4.41
A_15_P245326			down	4.25	11.41
A_15_P181996			down	2.67	2.79
A_15_P180711	LOC564655	XM_001921843	up	3.08	3.79
A_15_P385965			down	3.80	2.26
A_15_P109098	zgc:66286	AF434191	up	2.28	2.00
A_15_P104923			down	3.69	2.68
A_15_P161366	zgc:101810	NM_001007772	up	2.24	2.41
A_15_P145076	si:busm1-112p11.1	NM_001030081	down	2.18	2.68
A_15_P313936		CT650977	down	2.90	2.25
A_15_P353410		CT673553	down	4.78	2.87
A_15_P289901			down	3.38	5.84
A_15_P177736	ubr5	NM_001164394	down	2.25	2.48
A_15_P103192	foxi1	NM_181735	up	3.85	3.61
A_15_P451615	LOC100008030	XM_001346311	up	4.29	4.10
A_15_P118474	LOC556422	XM_679236	up	2.88	2.02
A_15_P166866	ttll6	NM_001105524	down	3.00	3.10
A_15_P543067			down	2.97	2.93
A_15_P115391	zgc:165343	NM_001080178	up	3.52	2.59

A_15_P105740		BI880356	down	2.13	2.23
A_15_P119120	sh3gl1b	NM_201497	down	2.17	2.17
A_15_P192346	si:ch211-244p18.3	NM_001045119	down	2.87	3.64
A_15_P110858	zgc:100942	NM_001003626	down	2.19	2.02
A_15_P286426			down	2.76	3.87
A_15_P204216			up	2.20	2.11
A_15_P291636		XM_001333200	down	2.33	4.37
A_15_P103929	zgc:92480	NM_001003485	up	2.60	2.20
A_15_P185721	rhcg2a	NM_001102618	up	3.29	2.31
A_15_P117430			up	2.74	3.54
A_15_P489717	alx4	XM_001340930	up	4.61	3.67
A_15_P166516	LOC561765	BC155848	up	4.45	2.79
A_15_P420305			down	2.39	4.31
A_15_P534667			up	2.07	2.01
A_15_P197596	LOC799375	XM_001339703	up	5.81	5.02
A_15_P461055	LOC796235	XM_001336503	up	4.26	9.34
A_15_P410375	mxra8a	NM_001080582	down	2.14	3.33
A_15_P185706			down	4.43	2.34
A_15_P414300	pcsk5b	NM_001083829	down	2.61	2.86
A_15_P106187	zgc:92074	NM_205743	down	2.47	2.36
A_15_P101148			down	2.44	3.59
A_15_P217756		EB839799	down	5.07	12.93
A_15_P120947			up	3.50	3.28
A_15_P170381	ism2	NM_001020509	down	2.61	3.98
A_15_P179471	LOC100001772	BC152007	down	2.07	2.61
A_15_P441185			up	2.69	2.52
A_15_P105231	LOC795659	BC095223	up	3.02	3.23
A_15_P158421	LOC100001854	BC092865	up	16.76	5.07
A_15_P600452			down	8.65	8.73
A_15_P192226	jag2	NM_131665	down	2.49	2.20
A_15_P302331	wu:fe05a04	XM_001345774	down	3.36	4.73
A_15_P197391	vrk2	NM_201170	down	3.25	2.50
A_15_P212351		BC094972	down	3.12	3.28
A_15_P166006			down	4.63	4.14
A_15_P259416			up	3.17	2.50
A_15_P238176			up	6.23	6.46
A_15_P152346	atp2a2a	NM_200965	up	3.28	2.20
A_15_P315396		CT618869	up	3.26	4.19
A_15_P189876	si:dkey-220o5.4	BC093315	up	3.46	2.42
A_15_P109217		BC122103	up	2.29	2.23
A_15_P167701		XM_001921641	down	2.20	2.38
A_15_P133346	cryabb	NM_001002670	up	6.86	3.08
A_15_P105778	igfbp1a	NM_173283	up	2.76	2.26

A_15_P145431	ca4a	NM_001114407	down	2.64	6.08
A_15_P115912			down	2.01	2.37
A_15_P101034	snn	NM_213046	down	2.37	3.52
A_15_P106750		CT668573	down	2.36	2.48
A_15_P388595		CT617386	down	4.85	2.48
A_15_P541757			up	2.31	2.27
A_15_P166661	zgc:172248	NM_001113599	up	2.76	2.44
A_15_P218851	efnb1	CU638799	down	2.82	8.32
A_15_P150026	rbm25	NM_199790	down	5.51	19.53
A_15_P130831	hoxa10b	NM_131155	up	2.28	2.04
A_15_P101168	t2gtl56	AB175329	down	4.70	7.27
A_15_P161406	zgc:194556	NM_001023572	down	2.26	2.02
A_15_P119205	igf2bp3	NM_131491	down	2.27	2.51
A_15_P186641	LOC570897	NM_001114441	up	3.49	2.89
A_15_P105544	smyd1b	NM_001039636	up	2.13	2.25
A_15_P195951	si:ch211-196l7.3	NM_001083096	down	2.39	2.68
A_15_P370975		CT612918	down	3.90	2.27
A_15_P430940			up	4.24	3.60
A_15_P335334	si:dkey-85n7.4	XM_691298	up	3.72	3.02
A_15_P514827	anxa1b	NM_181759	up	3.66	3.34
A_15_P113289	jph1b	NM_001044348	down	4.78	6.65
A_15_P539282			down	3.77	2.66
A_15_P113646			down	3.53	4.82
A_15_P138231	si:dkey-121a9.3	NM_001099256	up	2.84	3.08
A_15_P268831	CH211-259K16.3	XM_684103	down	3.37	6.58
A_15_P443355			down	3.86	2.98
A_15_P104637			up	2.84	2.00
A_15_P101217	acta1	AI964279	down	2.92	2.22
A_15_P109872		DQ869310	down	2.88	2.64
A_15_P189576			down	2.53	3.11
A_15_P308616	sb:cb69	CB333781	up	3.17	2.25
A_15_P303071	LOC570082	XM_693515	down	4.23	5.48
A_15_P197751	nin	BC135044	down	2.22	3.39
A_15_P100200		BC083467	down	5.80	4.06
A_15_P184081		BC090739	down	2.02	4.17
A_15_P107314	hoxb6a	NM_131119	down	2.14	2.12
A_15_P239316	LOC566775	XM_001922115	up	2.27	2.91
A_15_P157041		BC091815	down	2.79	3.33
A_15_P110740			down	57.61	9.62
A_15_P176766	zgc:165650	NM_001100149	down	2.92	3.00
A_15_P275951			up	2.05	2.27
A_15_P113217	orai1	NM_205600	down	3.31	3.25
A_15_P176941	ktn1	NM_200081	down	3.72	3.44

A_15_P137336			down	2.45	3.13
A_15_P341850	lin37	NM_001017888	up	2.22	2.17
A_15_P113584			up	2.65	2.15
A_15_P375100	si:ch211-240l19.7	NM_001082929	up	2.36	4.01
A_15_P112482			up	2.35	2.01
A_15_P119038	myf6	NM_001003982	up	7.70	4.58
A_15_P170951	zgc:163000	NM_001082864	up	6.91	6.66
A_15_P110719	slc4a4b	XM_685028	down	3.24	4.27
A_15_P450585			down	2.38	2.32
A_15_P110061			up	2.97	3.90
A_15_P597232	wu:fk57a03	EH995454	down	3.77	2.13
A_15_P141581	zgc:136571	NM_001045263	down	4.08	3.65
A_15_P302581			down	6.70	4.73
A_15_P146401	LOC568671	XM_692016	up	4.15	3.45
A_15_P219476	slc2a15b	NM_001020494	up	3.00	3.43
A_15_P354645		CT693264	down	2.45	2.44
A_15_P147096	zfr	NM_199558	down	3.09	4.87
A_15_P603772			down	2.44	2.16
A_15_P177306	taf3	NM_001042744	down	2.07	2.72
A_15_P206906	wnt7a	NM_001025540	up	4.36	5.38
A_15_P221496	foxq1	NM_212907	up	3.83	3.86
A_15_P134221	kiaa0261	BC090473	down	3.62	3.18
A_15_P475655			up	3.21	2.58
A_15_P272306	cpb1	NM_001110021	up	8.79	8.35
A_15_P379795	fancl	NM_212982	up	2.04	2.79
A_15_P113865	grid2	NM_001004123	down	2.59	4.27
A_15_P208301	atf7a	NM_001030205	down	2.62	4.61
A_15_P197381	LOC557043	XM_680019	up	3.51	2.47
A_15_P134551	thoc2	NM_001003847	down	3.24	3.18
A_15_P140511	gnptab	BC133107	down	2.72	2.91
A_15_P199896		BC057484	down	2.23	3.72
A_15_P179111	LOC792773	BC151895	down	2.36	2.67
A_15_P172146	si:dkey-121a9.3	NM_001099256	up	3.04	2.75
A_15_P155686	zgc:172197	NM_001114925	down	2.63	2.41
A_15_P115524	LOC559981	XM_683371	down	2.29	3.35
A_15_P141721	zgc:113363	NM_001123302	down	2.38	2.32
A_15_P601437			down	2.22	4.84
A_15_P221646			down	6.72	6.68
A_15_P115933	unk	NM_200236	down	3.43	2.12
A_15_P322176			down	2.45	2.15
A_15_P134501	arl13b	NM_173272	down	2.73	3.67
A_15_P441355			down	4.85	2.37
A_15_P520792			up	2.77	3.39

A_15_P350655			down	3.07	2.93
A_15_P162651	tmem54	NM_001044716	down	3.64	7.54
A_15_P118446	zgc:123203	NM_001037580	up	6.97	3.55
A_15_P352405		CT677687	down	3.86	2.16
A_15_P185481	LOC100148397	BC134195	down	3.85	3.58
A_15_P119760	LOC100149829	BC091928	up	3.31	2.76
A_15_P374845			up	2.12	2.42
A_15_P161051	zgc:153278	NM_001045434	down	2.11	2.77
A_15_P131656	cel.2	AF003943	up	2.06	3.37
A_15_P495822			up	8.70	4.66
A_15_P490582	LOC793282	XM_001332970	down	3.83	4.71
A_15_P472310			down	2.00	2.18
A_15_P579977			down	2.24	2.10
A_15_P144621		XM_001919509	down	5.02	11.58
A_15_P137721		XM_692723	down	2.76	3.31
A_15_P167016	LOC799514	BC124167	down	2.10	4.92
A_15_P205136	cldnb	NM_131763	up	2.46	2.78
A_15_P211841	gadd45b	NM_213031	up	6.98	3.06
A_15_P232456			down	3.16	2.14
A_15_P109820	si:ch211-51m24.3	NM_001044858	down	2.04	3.29
A_15_P117020	tcf12	NM_214816	down	2.63	2.77
A_15_P111428	traf7		up	2.32	2.36
A_15_P157371	LOC553505	BC092720	down	2.15	3.07
A_15_P119804	zgc:136894	NM_001045303	down	2.06	2.86
A_15_P102042		CF266409	down	2.55	4.31
A_15_P255186		CT594166	down	2.16	2.32
A_15_P401760			down	3.23	2.43
A_15_P103720			up	4.04	2.94
A_15_P186911	LOC556557	XM_679384	up	8.24	5.43
A_15_P198746		BC090523	down	3.09	3.29
A_15_P154551	LOC100148585	XM_001921436	down	2.12	3.27
A_15_P237781	zgc:163064	NM_001089510	down	2.82	3.77
A_15_P530182			up	2.33	2.81
A_15_P312580			down	2.78	2.51
A_15_P198841	myom1a	NM_001161340	down	2.11	4.73
A_15_P112737			up	3.24	2.15
A_15_P512792	im:7158733	XM_001922324	up	2.10	2.02
A_15_P106284	wasla	NM_001083006	down	2.40	2.22
A_15_P384995		CT625844	down	3.20	2.09
A_15_P157636		XM_687425	up	3.72	2.13
A_15_P306181			up	2.79	2.39
A_15_P469045			down	7.06	5.21
A_15_P311906	marveld2b	NM_001126406	up	2.18	2.58

A_15_P104834	zgc:162825	NM_001089526	up	2.98	3.96
A_15_P104962			down	2.95	3.03
A_15_P452665		EB974833	up	5.37	5.82
A_15_P188116	zgc:66419	NM_201111	down	3.44	4.64
A_15_P179656	zgc:174938	NM_001105700	up	2.90	4.15
A_15_P329971		CT694479	up	4.22	4.09
A_15_P316916	slc38a4	NM_001005944	down	6.37	5.68
A_15_P286291	si:ch211-196f5.2	NM_001044802	up	4.48	4.19
A_15_P213161	zgc:153663	BC122368	down	11.76	36.04
A_15_P102457	LOC565839	XM_689101	down	2.05	2.95
A_15_P562167	LOC100149829	BC091928	up	7.26	6.44
A_15_P176046	zgc:112303	NM_001030159	down	2.43	2.24
A_15_P336340	zgc:66117	NM_199560	up	2.60	2.25
A_15_P206781	p2rx3b	NM_198986	up	2.29	2.82
A_15_P310341	LOC100006622	XM_001345278	up	2.74	3.04
A_15_P114825	zgc:112450	NM_001020700	down	2.80	2.06
A_15_P422405			down	5.56	6.81
A_15_P203556	ezrl	NM_001020490	down	2.36	6.08
A_15_P340035			down	4.75	2.23
A_15_P597172	LOC100149324	XM_001920205	up	3.25	3.38
A_15_P101232	slc31a1	NM_205717	down	2.00	2.28
A_15_P171671	slc1a4	NM_001002513	up	3.66	4.69
A_15_P180531	zgc:101080	NM_001003568	up	2.11	2.38
A_15_P177431		BC122132	down	2.99	3.99
A_15_P176771	cx30.9	NM_001007288	up	2.83	2.56
A_15_P289331			up	2.71	2.70
A_15_P201081	LOC100005060	XM_001344175	up	2.42	2.23
A_15_P243556	LOC100000365	XM_001340547	down	2.13	3.43
A_15_P113167	zgc:110243	NM_001017629	up	4.14	3.00
A_15_P331599	pdxdc1	NM_001004552	down	2.74	4.56
A_15_P102501	rbmx2	NM_001025166	down	2.20	3.10
A_15_P440935			up	5.67	4.14
A_15_P577242			down	2.66	2.08
A_15_P162786	rab11fip4a	NM_001002533	up	4.55	2.68
A_15_P150916	zgc:112062	NM_001020648	up	2.68	2.63
A_15_P564217		BC124727	up	2.41	2.17
A_15_P139796	kcnj1	NM_201035	up	2.17	2.41
A_15_P116658	zgc:154064	NM_001077726	down	3.14	3.25
A_15_P160521	zgc:73328	NM_199686	down	2.75	3.45
A_15_P111884	exorh	NM_131212	up	2.70	2.66
A_15_P442195		EG579885	down	4.63	3.97
A_15_P103952	atad1a	NM_001007112	up	2.33	3.65
A_15_P100336		CT679047	down	5.07	2.95

A_15_P325921		CT697531	down	3.19	2.98
A_15_P186821	LOC795768	XM_001335992	up	7.33	8.53
A_15_P115509			up	3.14	2.96
A_15_P178796	zgc:165467	NM_001103113	down	2.18	4.74
A_15_P120563	cacna2d1a	NM_001044960	down	3.94	3.77
A_15_P119415	socs3a	NM_199950	up	2.80	2.28
A_15_P228476	LOC100150267	XM_001921702	down	2.23	3.06
A_15_P168206			down	3.65	2.00
A_15_P193936	zgc:162897	NM_001098195	down	2.40	2.10
A_15_P338920			down	2.36	2.25
A_15_P321971	ubtfl	NM_201003	down	2.68	2.33
A_15_P207846	si:dkey-19f23.2	NM_001044907	up	2.17	2.40
A_15_P108976	zgc:174574	NM_001115070	up	3.86	3.02
A_15_P164201	slc39a1	NM_212583	up	3.54	4.55
A_15_P312831		CT613529	down	6.65	4.21
A_15_P599207	ela3l	NM_001024408	up	2.19	2.20
A_15_P146986	flot2a	NM_213075	down	2.03	3.89
A_15_P141146	zgc:158773	NM_001082411	up	2.85	3.79
A_15_P168596		BC090480	down	3.09	4.21
A_15_P203526	LOC557381	XM_680435	up	2.87	2.14
A_15_P350610		XM_689701	down	2.34	2.00
A_15_P168231	cd82	NM_212663	up	2.10	2.39
A_15_P112922		BC059615	down	3.09	3.63
A_15_P120851	c6ast3	NM_001013526	up	2.02	2.84
A_15_P259631	zgc:65997	NM_200553	up	2.45	2.11
A_15_P104211	sh3glb2	NM_201119	up	2.60	2.32
A_15_P333284	si:ch211-199c19.3	XM_686942	down	3.89	4.50
A_15_P180446	CH211-233A1.7	NM_001161350	up	2.01	4.26
A_15_P383965			down	2.06	2.74
A_15_P192211			down	3.23	3.17
A_15_P472535	zgc:163064	NM_001089510	down	2.52	3.16
A_15_P109891			down	4.20	3.47
A_15_P110159	ela2l	AY179345	up	4.66	3.93
A_15_P116155	krt15	NM_213523	up	2.91	2.68
A_15_P101150	zgc:77306	NM_213228	up	2.91	2.04
A_15_P560767		CT588033	down	5.35	12.73
A_15_P548707			down	2.23	4.04
A_15_P153756	snai3	NM_001077385	up	2.54	2.13
A_15_P175206	anxa1b	NM_181759	up	2.29	2.37
A_15_P351880		CT692096	up	2.87	3.17
A_15_P407970	LOC794711	XM_001334684	up	2.73	4.12
A_15_P184621		BC083371	down	3.31	5.55
A_15_P102966	zgc:64012	NM_214761	down	2.55	2.13

A_15_P111240		BC083288	down	2.25	2.81
A_15_P366150	dgat2	NM_001030196	down	2.02	2.27
A_15_P114940	zgc:163073	NM_001089511	up	5.19	3.53
A_15_P159131	si:dkey-108d22.2	NM_001126110	up	4.49	2.85
A_15_P120310			up	2.22	2.36
A_15_P474630	letm2	BC154838	down	2.50	3.23
A_15_P104132	zgc:112165	NM_001020656	up	4.01	4.09
A_15_P114316	si:dkey-86e18.1	NM_001025491	up	2.09	2.28
A_15_P119580	zgc:174342	NM_001110288	up	3.34	5.28
A_15_P132526	klf4	NM_131723	up	4.92	4.26
A_15_P578332	LOC557353	XM_001335696	down	2.77	4.36
A_15_P110703			up	2.76	3.98
A_15_P222826		BC135030	down	2.20	2.82
A_15_P448290	ddx5	NM_212612	down	2.14	3.13
A_15_P321361			down	2.62	2.11
A_15_P150506	zgc:158393	NM_001080016	up	4.05	2.13
A_15_P589482		EB971273	up	4.26	3.48
A_15_P221436	LOC100151124	XM_001921076	down	2.79	9.61
A_15_P309491	LOC558375	XM_681577	down	3.12	3.27
A_15_P184246		EB930870	down	2.80	3.18
A_15_P521517	zgc:163000	NM_001082864	up	4.01	3.94
A_15_P565127			up	3.60	3.49
A_15_P506462	LOC563316	BC155650	down	2.07	2.78
A_15_P335519	si:ch211-251j10.4	NM_001123232	down	10.11	16.65
A_15_P274421			up	5.82	2.48
A_15_P177096	spef2	NM_001128232	down	2.34	2.37
A_15_P180186			down	3.06	3.03
A_15_P180171	LOC561649	XM_685063	down	2.49	4.54
A_15_P117786	zgc:110045	NM_001039984	down	2.70	2.05
A_15_P457045		XM_001923765	down	3.21	3.47
A_15_P167123	LOC100148026	XM_001919814	up	2.82	3.95
A_15_P221461	snapc1a	NM_200263	up	3.62	3.01
A_15_P106753			up	3.23	2.57
A_15_P146841		XM_691963	up	3.45	3.06
A_15_P160541	LOC562928	XM_686293	up	4.65	3.61
A_15_P110814	nos2a	NM_001104937	up	3.81	3.45
A_15_P288711			up	2.93	2.89
A_15_P309521			up	3.23	2.63
A_15_P112683	cdca8	NM_001007456	up	3.38	3.64
A_15_P197131	socs5b	NM_001113797	down	6.46	6.51
A_15_P196681			up	5.41	4.98
A_15_P138376	LOC565670	XM_688937	down	2.66	4.78
A_15_P201066		AY648770	down	3.47	3.28

A_15_P252566		CR929752	down	3.62	3.73
A_15_P494522	acin1b	BC045312	down	2.32	3.12
A_15_P212121	tln1	NM_001009560	down	2.49	2.15
A_15_P116696			down	2.56	2.32
A_15_P261491	LOC100004986	XM_001344114	up	3.49	2.24
A_15_P104101	cbln2a	NM_001128688	up	4.92	4.72
A_15_P505967		XM_001921567	down	2.81	5.15
A_15_P155191	LOC798730	XM_001339126	up	7.38	6.33
A_15_P200761		CK015821	up	5.03	6.70
A_15_P510572		XM_680968	down	2.85	7.20
A_15_P104006			down	2.71	2.48
A_15_P119855	prkcb1l	NM_201029	up	3.58	4.62
A_15_P373835	eif2ak4	XR_044737	down	2.98	3.08
A_15_P392800	fstb	NM_001109831	up	2.07	2.92
A_15_P224261			down	2.11	2.19
A_15_P112944	map2k1	NM_213419	down	2.05	2.03
A_15_P160046	LOC100149074	XM_001919536	down	2.77	2.70
A_15_P579427	zgc:91908	NM_001002179	up	5.87	3.00
A_15_P419550	gch2	NM_131667	up	4.68	3.79
A_15_P471315			down	7.63	6.98
A_15_P100412	tbx1	NM_183339	up	2.57	2.22
A_15_P208351	zgc:113263	NM_001013331	down	2.38	4.65
A_15_P135191		XM_001334548	down	3.14	2.71
A_15_P327981		CT730864	down	3.38	2.46
A_15_P153666	klf4	NM_131723	up	2.32	2.09
A_15_P260731		CA474667	up	2.45	2.06
A_15_P110458	jph1a	NM_001166256	down	3.34	2.77
A_15_P591092	wu:fb53f04	EH580949	up	2.49	2.25
A_15_P547357		CO924904	up	5.86	6.12
A_15_P104403	pawrl	NM_001006015	down	2.56	3.73
A_15_P190396			up	2.44	2.66
A_15_P118636	sf3a1	NM_200094	down	3.56	6.41
A_15_P588067		CK703328	down	5.71	2.75
A_15_P113973			up	3.64	2.17
A_15_P512452			down	2.46	2.24
A_15_P177561	si:ch211-274p22.2	BC122423	down	2.90	4.67
A_15_P111401	zgc:153154	NM_001045413	up	3.38	5.49
A_15_P541997	LOC100150319	EH581399	down	2.75	2.01
A_15_P119822	oclnb	NM_001008618	up	2.51	2.32
A_15_P472735	si:dkey-274c14.3	XM_682770	down	3.22	2.93
A_15_P184096	ppat	NM_001082877	up	2.65	2.30
A_15_P142331	myf6	NM_001003982	up	3.43	2.76
A_15_P113127	grhl1	XM_001923728	up	2.19	2.01

A_15_P435980		CA787485	down	3.32	3.38
A_15_P557417	LOC100150225	XM_001921222	down	3.25	2.41
A_15_P115666	zgc:153764	NM_001077318	up	3.36	2.21
A_15_P109666	zgc:136396	NM_001040291	up	2.01	3.27
A_15_P104423	dctn1a	NM_001077212	down	3.11	4.15
A_15_P121130			down	2.25	4.82
A_15_P529307	zgc:162611	NM_001089517	up	3.76	3.13
A_15_P201106		DQ812123	down	2.97	3.27
A_15_P155871		XM_001345293	up	2.14	2.38
A_15_P177246	LOC560667	BC115075	down	2.01	2.50
A_15_P241836	LOC799182	XM_001339531	up	2.43	2.15
A_15_P407150	wu:fb98b04	CT597797	down	2.62	4.46
A_15_P112191	cx35.4	NM_001017685	up	4.08	4.43
A_15_P190611	LOC559763	XM_001922074	up	3.91	2.93
A_15_P249136			up	2.75	2.77
A_15_P178211	si:ch211-218c6.1	NM_001044339	down	3.10	4.31
A_15_P103991	zgc:112426	NM_001017793	up	5.42	6.07
A_15_P191386			up	3.67	4.66
A_15_P178181	zgc:153452	NM_001077569	down	2.06	3.53
A_15_P321036			up	3.43	4.25
A_15_P366130	rnf24	NM_001007352	down	2.55	2.36
A_15_P115079	rcor2	NM_205638	down	2.58	2.41
A_15_P199886	prkag3	BC090443	up	2.44	—
A_15_P407165		AI601465	down	9.59	—
A_15_P518272			down	2.69	—
A_15_P225241			up	2.03	—
A_15_P313636		CT628855	down	3.24	—
A_15_P508722			down	2.37	—
A_15_P563252		CT602888	down	2.29	—
A_15_P350070	mcat	NM_001045043	up	2.26	—
A_15_P102647	zgc:158393	NM_001080016	up	3.21	—
A_15_P114781	mstn	NM_131019	up	2.25	—
A_15_P334054			down	2.63	—
A_15_P101674	si:dkeyp-73d8.6	NM_001130644	down	2591.71	—
A_15_P282306	bmpr1ab	NM_001004585	up	2.19	—
A_15_P530997			down	2.71	—
A_15_P199126	zgc:195172	NM_001128817	up	2.94	—
A_15_P341740			down	2.54	—
A_15_P166841			up	2.39	—
A_15_P198881	puf60a	NM_001105588	up	2.38	—
A_15_P103560	zgc:77082	NM_212870	down	2.42	—
A_15_P590117		CT686697	down	2.04	—
A_15_P239856	zgc:65827	NM_213155	up	2.43	—

A_15_P109797	zgc:85975	NM_213265	up	2.02	—
A_15_P286856			down	2.39	—
A_15_P110111			down	4.32	—
A_15_P160286		XM_001921251	down	2.07	—
A_15_P114160	lmnb1	NM_152972	down	2.16	—
A_15_P362945			up	2.04	—
A_15_P225216			down	2.28	—
A_15_P566942			up	3.01	—
A_15_P119390			down	4.35	—
A_15_P100437			down	9.74	—
A_15_P139291	LOC100002779	XM_001342445	down	4.45	—
A_15_P426445		CT585723	down	2.40	—
A_15_P353120			down	2.16	—
A_15_P393105		CT696504	up	2.33	—
A_15_P515372			up	2.02	—
A_15_P486250			down	2.26	—
A_15_P141421	si:dkey-173l11.4	NM_001077575	up	2.90	—
A_15_P435485	im:7145045	XM_687130	down	2.03	—
A_15_P479160		CT724746	down	2.60	—
A_15_P163896	LOC556956	NM_001034972	up	2.87	—
A_15_P212286	LOC100004140	BC091954	down	4.14	—
A_15_P250021		CT635404	down	2.62	—
A_15_P531307	zgc:162600	NM_001089486	up	2.08	—
A_15_P377775	LOC553259	BC121769	up	4.76	—
A_15_P590177		CT676876	down	2.09	—
A_15_P366600	gosr2	NM_199688	up	2.17	—
A_15_P172931	cx41.8	NM_001034988	down	2.02	—
A_15_P505992			down	2.38	—
A_15_P592467			down	2.25	—
A_15_P301966			down	2.44	—
A_15_P183381	tpd52l2b	NM_199582	up	2.70	—
A_15_P119121		BQ109731	down	2.43	—
A_15_P487450			down	2.10	—
A_15_P176021	dync1li1	NM_001102428	up	2.31	—
A_15_P243881			down	2.26	—
A_15_P100253		BC071301	down	2.28	—
A_15_P116856	taf1	NM_001044785	down	2.08	—
A_15_P225096	si:dkey-7b17.2	BC134873	up	2.00	—
A_15_P148176	CH211-157C24.2	NM_001128329	down	2.39	—
A_15_P193921	wdr92	NM_001017621	down	3.51	—
A_15_P105276		BC068437	up	2.01	—
A_15_P264946		AW059303	down	3.19	—
A_15_P285036	LOC100150476		down	2.15	—

A_15_P163011	foxp3	NM_001082874	down	2.02	—
A_15_P252436		CR928168	down	9.03	—
A_15_P105113		CK360741	down	2.72	—
A_15_P172551	optn	NM_001100066	up	2.02	—
A_15_P358615		CT685142	down	2.30	—
A_15_P576277			down	2.48	—
A_15_P458110	tmem176l.4	NR_023331	down	3.55	—
A_15_P180916	zgc:110610	BC095333	down	2.38	—
A_15_P275856			down	2.76	—
A_15_P192311	slain2	BC125960	down	2.29	—
A_15_P423950		CT708980	down	2.16	—
A_15_P103460	arl4cb	NM_213248	up	2.60	—
A_15_P552352			down	2.90	—
A_15_P533267			down	2.43	—
A_15_P512892			up	3.49	—
A_15_P315136		CT604282	down	2.63	—
A_15_P104563		XM_693146	up	2.44	—
A_15_P121481			up	2.06	—
A_15_P185556	raver2	XM_001338047	up	2.96	—
A_15_P387680			down	3.10	—
A_15_P109920			up	2.98	—
A_15_P224431	si:ch211-67e16.9	NM_001104938	up	2.42	—
A_15_P533157			down	2.04	—
A_15_P179241	zgc:103624	BC152157	down	2.26	—
A_15_P121072	mcat	NM_001045043	up	2.31	—
A_15_P373380		CT721059	down	2.01	—
A_15_P179696		BC152020	down	3.16	—
A_15_P137784			up	2.14	—
A_15_P188256	LOC571064	XM_694618	down	2.64	—
A_15_P410615	LOC566978	EE691164	up	2.20	—
A_15_P184651	zgc:114082	NM_001039977	down	4.38	—
A_15_P134146	cdk5	BC085381	down	2.00	—
A_15_P439700			down	2.23	—
A_15_P349040			down	2.51	—
A_15_P193791	bbc3	NM_001045472	up	2.38	—
A_15_P594182	si:dkey-242k1.6	XM_001337567	down	2.02	—
A_15_P158041	LOC567806	BC146707	up	2.86	—
A_15_P162181	zgc:153288	NM_001077321	down	255.83	—
A_15_P593682			up	2.60	—
A_15_P495807			up	2.30	—
A_15_P244181	hhatlb	NM_001077390	up	3.73	—
A_15_P197426	ap3m1	NM_201292	up	2.07	—
A_15_P533684			down	2.05	—

A_15_P110005	zgc:110380	NM_001017660	up	2.46	—
A_15_P416150			up	2.20	—
A_15_P598182		BM070936	down	2.69	—
A_15_P165651	LOC563339	XM_686703	down	2.04	—
A_15_P277051			down	2.05	—
A_15_P106768	mrpl3	NM_200007	up	2.29	—
A_15_P509662	puf60a	NM_001105588	up	2.43	—
A_15_P104220	DKEY-30O19.2	NM_001123236	up	2.66	—
A_15_P522977		CT716541	down	3.23	—
A_15_P387300		CT615859	down	2.18	—
A_15_P105487			down	2.17	—
A_15_P225846			down	2.16	—
A_15_P288781			down	2.14	—
A_15_P391965	blcap	NM_131493	up	2.16	—
A_15_P117149			down	2.12	—
A_15_P242031	LOC563377	XM_686740	down	2.09	—
A_15_P130541	fgf1	NM_200760	up	2.15	—
A_15_P107392	zgc:153615	NM_001077791	up	2.26	—
A_15_P167336	polr3e	NM_212754	up	7.57	—
A_15_P147146	DKEY-19F4.1	XM_001919526	down	2.15	—
A_15_P139951	nrxn1b	NM_001079959	down	2.23	—
A_15_P164901			up	2.01	—
A_15_P203136		XM_692343	down	3.13	—
A_15_P152026	her7	NM_131609	down	2.36	—
A_15_P239846			up	2.48	—
A_15_P423075	eif6	NM_200944	up	2.02	—
A_15_P298926		EB980707	down	2.26	—
A_15_P423170			down	2.14	—
A_15_P265881	LOC566587	XM_689863	up	2.13	—
A_15_P101660	zgc:153218	NM_001077155	up	2.54	—
A_15_P110003	cradd	NM_001006066	up	2.01	—
A_15_P214871			down	2.15	—
A_15_P143181	zgc:66430	NM_200055	up	2.56	—
A_15_P470595	ube2i2	NM_131833	up	2.01	—
A_15_P121322	ndrg3b	NM_199797	up	2.16	—
A_15_P184116	moxd1	NM_001045206	up	2.21	—
A_15_P107760	si:dkey-39n1.2	NM_001128364	up	2.58	—
A_15_P203401			down	3.21	—
A_15_P492102		XM_691455	up	2.05	—
A_15_P110105			up	2.44	—
A_15_P181011	LOC100004941	XM_001344083	up	2.52	—
A_15_P291611		BC054690	up	3.32	—
A_15_P326876			down	2.01	—

A_15_P196411	ndrg1l	NM_200692	up	2.75	—
A_15_P116417	rbm41	NM_001007405	down	2.40	—
A_15_P118948	rsad1	NM_001083557	down	2.35	—
A_15_P152571	map4k2l	NM_001040358	down	2.15	—
A_15_P180741	htr1ab	NM_001145766	up	2.12	—
A_15_P360905		CT643967	down	2.48	—
A_15_P349625			up	2.23	—
A_15_P176546		BC134078	down	2.70	—
A_15_P119061	fgl2	NM_001025539	down	2.67	—
A_15_P516297	ube2i2	NM_131833	up	2.10	—
A_15_P217176	ndor1	NM_200648	up	2.19	—
A_15_P183421	LOC567047	FJ469678	up	2.48	—
A_15_P574827		EH552365	down	2.87	—
A_15_P545722	thoc5	NM_212692	up	2.09	—
A_15_P161711	zgc:158706	NM_001080201	up	2.04	—
A_15_P182891	LOC571066	XR_044837	down	2.25	—
A_15_P198631	si:dkey-218h11.4	NM_001044972	up	2.02	—
A_15_P160826	zgc:153654	NM_001077169	up	2.36	—
A_15_P105018	LOC564564	XM_685001	up	2.47	—
A_15_P399890			up	2.91	—
A_15_P131351	ndel1a	NM_201344	up	2.05	—
A_15_P289551			down	4.17	—
A_15_P187236		XM_678984	down	3.42	—
A_15_P114935	wu:fb78c02	XM_001923468	up	2.12	—
A_15_P115764	ampd3	NM_199848	up	2.00	—
A_15_P393170		BE016846	down	2.12	—
A_15_P192116	rxfp3	XM_001346785	up	4.93	—
A_15_P544722			down	2.02	—
A_15_P100713		BC154050	up	4.93	—
A_15_P113091	ntn1b	NM_130998	down	2.10	—
A_15_P131901	rnf2	NM_131213	up	2.43	—
A_15_P241431	LOC100007939	XM_001346237	down	2.71	—
A_15_P189111	DKEY-190G11.2	XM_690282	down	4.81	—
A_15_P113984	rfc5	NM_001003862	up	2.20	—
A_15_P154371	tspan7b	NM_001130818	up	4.52	—
A_15_P542567			down	4.50	—
A_15_P192521	hspb6		down	2.33	—
A_15_P141101	ndufv1	NM_001003747	up	2.01	—
A_15_P105389	zgc:152779	NM_001077535	up	2.12	—
A_15_P284621	wu:fb94b04	XM_685394	down	2.10	—
A_15_P186301	LOC559303	XR_028989	down	2.10	—
A_15_P418925			down	2.19	—
A_15_P157906	zgc:86773	NM_001002129	up	2.33	—

A_15_P201911	mstn	BC094979	up	2.67	—
A_15_P100837	aldoc	NM_194384	up	2.34	—
A_15_P113621		XM_694722	down	3.66	—
A_15_P106129	epd	NM_131005	up	2.24	—
A_15_P120069			up	2.02	—
A_15_P103583		BM532658	down	5.02	—
A_15_P581627			up	2.64	—
A_15_P482580		EH454068	down	2.12	—
A_15_P538407			up	2.31	—
A_15_P113678	zgc:175088	NM_001113798	up	2.57	—
A_15_P117176	brf1	NM_199898	down	6.79	—
A_15_P187026	tmem86b	NM_001045368	up	2.23	—
A_15_P280866	trnau1apl	NM_001029966	down	2.10	—
A_15_P383370			up	2.07	—
A_15_P142136	mcfd2	NM_001005939	up	2.10	—
A_15_P345595	cct5	NM_212613	up	2.03	—
A_15_P109893	pik3ip1	NM_198374	up	2.06	—
A_15_P203796	zgc:175182	NM_001114410	up	2.48	—
A_15_P183581	klhl21	NM_207081	up	2.10	—
A_15_P403710	LOC565111	XM_001921567	up	2.47	—
A_15_P441080	LOC100149852	XM_001923886	down	4.80	—
A_15_P141291	si:dkeyp-31e2.1	NM_001082925	up	2.80	—
A_15_P481535		CT671392	down	4.77	—
A_15_P179416	zgc:171763	NM_001105696	down	2.12	—
A_15_P502402	got2a	NM_213379	up	2.43	—
A_15_P580012			down	2.53	—
A_15_P234801		EH582738	down	2.45	—
A_15_P110781			down	2.15	—
A_15_P360885		CT729768	down	2.40	—
A_15_P111326	rapsn	NM_178305	up	2.38	—
A_15_P120217	mut	NM_001099226	up	2.26	—
A_15_P112682		BC154795	up	2.63	—
A_15_P158911	CH211-136G2.1	BC154645	up	2.11	—
A_15_P100918	LOC407614	NM_001161351	down	2.64	—
A_15_P104186	zgc:112161	NM_001020655	up	2.95	—
A_15_P502977			down	2.71	—
A_15_P356670			down	12.33	—
A_15_P148711	zgc:153648	NM_001045446	down	2.06	—
A_15_P418670			down	2.18	—
A_15_P106404	si:ch211-14a17.7	NM_001007180	down	5.30	—
A_15_P115872			down	3.49	—
A_15_P210401		CT631366	down	4.46	—
A_15_P467840			up	2.36	—

A_15_P329236		CT653105	down	2.26	—
A_15_P303966	LOC796927	CK238742	down	2.03	—
A_15_P131921	rds2	NM_131566	up	2.36	—
A_15_P323881			up	4.04	—
A_15_P119305	si:ch211-67n3.4	NM_001144785	down	2.56	—
A_15_P117867			down	2.70	—
A_15_P500387			down	2.70	—
A_15_P188876	DKEY-83K24.4	NM_001130073	up	2.12	—
A_15_P137201	LOC559395	XM_682733	up	2.69	—
A_15_P179061	zgc:113377	BC152494	down	2.86	—
A_15_P140701	zgc:112437	NM_001017771	up	2.03	—
A_15_P567227	zgc:158345	NM_001083013	down	2.03	—
A_15_P106150	oxa1l	NM_001105130	up	3.73	—
A_15_P488015			down	2.58	—
A_15_P229696		EB960943	up	2.04	—
A_15_P360015		CT687338	down	3.86	—
A_15_P100729	lrrn1	NM_001130694	up	2.38	—
A_15_P113774	depdc6	NM_001077320	down	2.22	—
A_15_P306646			down	4.29	—
A_15_P434725	pgam5	NM_001007323	up	2.53	—
A_15_P175501	si:ch211-191d15.2	NM_001003632	up	2.07	—
A_15_P134654	zgc:162640	NM_001082814	up	2.16	—
A_15_P117269	atp2a1l	NM_001077533	up	2.33	—
A_15_P130206		BC154459	down	2.00	—
A_15_P594622		XM_001919953	down	2.11	—
A_15_P162256	kcnj11	NM_001039827	up	2.36	—
A_15_P179876	LOC100001767	CN177292	up	3.43	—
A_15_P325396		CT701933	down	2.12	—
A_15_P185506	erm	NM_131205	up	3.98	—
A_15_P114594	nrp1b	NM_205674	up	2.24	—
A_15_P154346			down	2.08	—
A_15_P100368	zgc:91968	NM_001002167	up	2.41	—
A_15_P104191			down	2.80	—
A_15_P111559	notch3	NM_131549	down	6.43	—
A_15_P177441		BC122152	up	3.16	—
A_15_P415805			up	3.08	—
A_15_P107868	zgc:73337	BC059655	up	84.37	—
A_15_P103567		BM035544	down	2.08	—
A_15_P404775		DN902661	down	2.48	—
A_15_P102168		AI545556	down	2.75	—
A_15_P119994	gpd1l	NM_001005934	down	2.67	—
A_15_P154736	ND3	CV489513	down	2.48	—
A_15_P193171	zgc:101644	NM_001008643	down	2.72	—

A_15_P120907	si:ch211-212d10.3	NM_001044843	up	2.22	—
A_15_P121335			down	2.07	—
A_15_P197036		XM_681566	up	2.41	—
A_15_P223521	LOC100150644	XM_001920798	down	3.11	—
A_15_P331101			up	3.86	—
A_15_P275301	casq2	NM_001002682	up	2.79	—
A_15_P146741	slc12a9	NM_001128548	up	2.33	—
A_15_P372115	pufa	XM_690488	up	2.05	—
A_15_P382560			down	2.18	—
A_15_P118680	gdf8l	NM_001004122	up	2.38	—
A_15_P111048	si:dkeyp-93c5.1	XM_681322	down	2.02	—
A_15_P193626	zgc:77051	NM_212866	up	2.07	—
A_15_P443465	LOC100004831	XM_001343998	down	2.50	—
A_15_P108830	cab39l1	NM_213501	down	2.51	—
A_15_P106128			down	2.22	—
A_15_P195161	zgc:158700	NM_001083031	up	2.12	—
A_15_P137806	LOC100008036		down	2.01	—
A_15_P170131	foxd3	NM_131290	up	2.08	—
A_15_P495617			down	2.29	—
A_15_P150381	capn2l	NM_001017807	up	2.07	—
A_15_P584722		BU710225	down	2.98	—
A_15_P560997		EH442032	down	2.22	—
A_15_P203336		BC129415	down	36.91	—
A_15_P117981	slc5a11	NM_001007300	down	3.09	—
A_15_P456500		BI846484	down	2.21	—
A_15_P212906	zgc:136827	NM_001040245	up	2.22	—
A_15_P110300	arglu1a	NM_213216	down	2.45	—
A_15_P185561	LOC794807	XM_001334793	down	2.93	—
A_15_P141256	zgc:109926	NM_001030076	up	2.85	—
A_15_P116098	golga5	NM_213411	up	2.25	—
A_15_P162226	zgc:113317	NM_001033725	down	212.56	—
A_15_P115904	zgc:77057	NM_214705	up	2.36	—
A_15_P503052			down	2.90	—
A_15_P120206	zgc:92034	NM_001004605	up	2.60	—
A_15_P120839	zgc:112221	NM_001020763	up	3.45	—
A_15_P347285			down	3.09	—
A_15_P531102			down	2.01	—
A_15_P314511		CT588715	down	2.21	—
A_15_P515327	zgc:92201	NM_001002640	up	2.15	—
A_15_P152307	eif2s1l	NM_199569	up	2.37	—
A_15_P361625		CT636357	down	2.43	—
A_15_P140386	zgc:154037	NM_001076584	up	2.08	—
A_15_P473805			down	2.39	—

A_15_P131666	gad1	NM_194419	up	2.31	—
A_15_P204026	sp8l	AY457141	up	2.03	—
A_15_P449250			down	4.01	—
A_15_P464860		AW059471	down	2.32	—
A_15_P509412	DKEY-20I10.7	XM_001336739	up	2.05	—
A_15_P114411	hars	NM_001004586	up	2.57	—
A_15_P114719	stc2	BC129138	up	2.12	—
A_15_P139481	prickle1b	NM_001030098	up	2.02	—
A_15_P109826			up	2.00	—
A_15_P106877	hsp47	NM_131204	down	2.63	—
A_15_P581397			up	2.24	—
A_15_P522742			down	2.39	—
A_15_P161486	si:dkey-24l11.4	NM_001025538	up	2.09	—
A_15_P111247	tp73	NM_183340	up	2.00	—
A_15_P526572			down	2.42	—
A_15_P107776			down	2.27	—
A_15_P112485	tyw3	NM_001002611	up	2.06	—
A_15_P481520		CT671245	down	2.79	—
A_15_P160661	cebpd	NM_131887	up	3.57	—
A_15_P554497		CO353902	down	2.44	—
A_15_P288581		BC163356	down	2.40	—
A_15_P111554	srfl	NM_200631	up	2.13	—
A_15_P551007	LOC100150557	EV756135	up	2.13	—
A_15_P552287	LOC556985	XM_679939	up	2.11	—
A_15_P292731	DKEY-219C10.5	NM_001115139	down	2.21	—
A_15_P243141			down	2.08	—
A_15_P105352	hoxc6b	NM_131530	down	2.22	—
A_15_P194741	ndrg4	NM_001045173	up	2.47	—
A_15_P376140	uros	NM_212828	up	2.64	—
A_15_P533307	zgc:66117	NM_199560	up	2.18	—
A_15_P520137	wu:fe48e11	BI889407	up	2.11	—
A_15_P166026	mpzl3	NM_213169	up	2.34	—
A_15_P153831	zgc:153919	NM_001045415	up	2.26	—
A_15_P178726		BC142921	down	2.16	—
A_15_P299287	minpp1	NM_201100	up	2.00	—
A_15_P168856	efr3a	NM_200827	down	2.41	—
A_15_P373490	LOC560275	XM_683672	up	2.03	—
A_15_P184141	aig1	XM_685654	up	2.64	—
A_15_P202191		BC095271	down	3.13	—
A_15_P362350			down	2.41	—
A_15_P302596			down	2.83	—
A_15_P577552	cebpd	NM_131887	up	3.32	—
A_15_P569897			down	2.20	—

A_15_P565902	eif6	NM_200944	up	2.43	—
A_15_P446290	rlbp1b	NM_205690	up	2.32	—
A_15_P198486	atp1b3a	NM_131221	up	2.24	—
A_15_P173636	zgc:165666	NM_001099242	down	2.10	—
A_15_P176986	si:dkey-162b3.6	NM_001161361	up	2.59	—
A_15_P152986	bnip3l2	NM_001012242	up	2.46	—
A_15_P473465			up	2.22	—
A_15_P107295		CT683426	down	2.20	—
A_15_P424295		CT675511	down	2.31	—
A_15_P191961	si:ch211-13n20.2	XM_693914	down	2.51	—
A_15_P102313			up	2.05	—
A_15_P312721		CT612545	down	2.38	—
A_15_P162141	prmt2	NM_001080175	down	2.22	—
A_15_P119403		BI845607	down	3.65	—
A_15_P154391	capzb	NM_199935	up	2.64	—
A_15_P493942	im:7149356	BC091903	down	2.13	—
A_15_P362575			down	2.94	—
A_15_P532367			down	51.69	—
A_15_P437545	smek1	NM_001044809	down	2.86	—
A_15_P370540	LOC100151593	CT735115	down	2.06	—
A_15_P119945			down	2.96	—
A_15_P167076	kif19	BC135048	down	—	6.26
A_15_P396485			down	—	2.91
A_15_P169926	zgc:158867	NM_001080611	down	—	2.65
A_15_P172961	zgc:163030	NM_001089413	up	—	7.09
A_15_P539822	flii	XM_683678	down	—	3.12
A_15_P140241	si:ch211-199m3.8	NM_001044849	down	—	2.28
A_15_P105293	zgc:91835	NM_001002201	down	—	3.30
A_15_P113120	serinc5	NM_213514	up	—	2.06
A_15_P119334	pdzk1ip1l	NM_001007760	down	—	2.55
A_15_P177991		BC129313	down	—	3.61
A_15_P394650		CK024856	down	—	2.39
A_15_P494432		BC151829	down	—	3.54
A_15_P105702	ubtf	NM_001005395	down	—	2.81
A_15_P111530	pdc2	NM_001025464	down	—	5.33
A_15_P109020	zgc:158446	NM_001083858	down	—	2.74
A_15_P111624	zgc:158803	NM_001089547	down	—	2.84
A_15_P500557			up	—	5.36
A_15_P270601	LOC795978	EH448452	up	—	2.21
A_15_P497057	DKEYP-20E4.6	NM_001164812	down	—	2.51
A_15_P164596	si:ch211-146f4.5	NM_001044998	down	—	4.02
A_15_P364875	cbl	NM_001007330	down	—	2.01
A_15_P306491			up	—	2.27

A_15_P186561	LOC556380	XM_679169	down	—	3.38
A_15_P503892	zgc:112104	NM_001017745	down	—	2.65
A_15_P527632		CT659012	down	—	2.42
A_15_P176976	pomgnt1	BC097123	up	—	2.05
A_15_P104848	tppp3	NM_201335	up	—	2.28
A_15_P205611	suv420h2	NM_001098186	down	—	2.02
A_15_P418090	si:ch211-173p18.3	NM_001044320	down	—	3.68
A_15_P189851	zgc:63688	NM_200838	down	—	2.31
A_15_P582052	LOC100004401	XM_001343682	down	—	28.95
A_15_P185251		XM_001919989	down	—	3.30
A_15_P196501	slc5a11	NM_001007300	down	—	2.22
A_15_P202781	ecd	NM_199891	down	—	2.36
A_15_P188771		XM_695839	down	—	2.89
A_15_P199021	zgc:100957	NM_001003620	down	—	2.47
A_15_P365320	cpne3	NM_200167	up	—	2.35
A_15_P100832		BM573563	up	—	2.20
A_15_P138091			up	—	2.00
A_15_P155861	sema6d	NM_212999	down	—	2.25
A_15_P234821		DR714895	down	—	2.23
A_15_P297801			up	—	2.12
A_15_P160076	zgc:194470	NM_001128765	down	—	2.34
A_15_P437720			up	—	2.28
A_15_P107489	top2b	NM_001045191	down	—	2.16
A_15_P443380			down	—	4.36
A_15_P173126	zgc:56095	NM_213013	up	—	2.20
A_15_P204612	zgc:136346	NM_001045276	down	—	3.08
A_15_P397760	atrxl	BC057486	down	—	2.67
A_15_P111171		XM_683165	down	—	4.96
A_15_P184941	mia3	NM_001105525	down	—	2.63
A_15_P145766	LOC561911	XM_685313	down	—	3.27
A_15_P550017	zgc:194294	NM_001128812	down	—	3.05
A_15_P178591		BC142767	down	—	2.36
A_15_P501087	DKEY-215K6.1	XM_679464	up	—	2.13
A_15_P380925			down	—	2.89
A_15_P156816	LOC100000778	XM_001337711	down	—	2.05
A_15_P188846	si:ch211-145m1.1	NM_001130781	down	—	2.01
A_15_P168911	zgc:152955	NM_001076613	down	—	2.23
A_15_P134141	taf5	NM_001037431	down	—	3.65
A_15_P201496	si:ch211-219a4.7	NM_001123233	down	—	2.66
A_15_P239436	phf8	XM_689807	down	—	2.69
A_15_P363695	zgc:153990	NM_001077271	up	—	2.27
A_15_P356510			down	—	3.19
A_15_P339915			down	—	2.61

A_15_P178171		BC134067	down	—	2.57
A_15_P446435	hic1l	NM_131483	down	—	2.05
A_15_P147446		XM_691602	down	—	2.63
A_15_P120887	zgc:56231	NM_213188	up	—	2.23
A_15_P181991		XM_684255	down	—	2.19
A_15_P235391	zgc:113259	NM_001014314	down	—	2.35
A_15_P110900	zgc:63528	NM_201032	down	—	2.05
A_15_P133611	ezrl	NM_001020490	down	—	3.87
A_15_P188781	si:dkey-49h9.6	BC091950	down	—	5.22
A_15_P170506	zgc:64187	NM_200417	down	—	2.40
A_15_P200271	LOC556634	XM_679483	up	—	2.31
A_15_P166806	dclre1a	NM_001020549	up	—	2.35
A_15_P444760	prtfdc1	NM_199518	up	—	2.06
A_15_P273176			up	—	2.09
A_15_P107158	usp40	BC054934	down	—	2.44
A_15_P192661	zgc:113418	NM_001012491	up	—	2.39
A_15_P204566	mtdhb	BC121767	down	—	2.12
A_15_P116413	zgc:171476	NM_001114886	down	—	2.12
A_15_P101831		BC076069	up	—	2.25
A_15_P208871	ptpra	NM_131888	down	—	2.19
A_15_P133426	smc4	NM_173253	down	—	2.38
A_15_P170016	aldh1a3	NM_001044745	up	—	2.46
A_15_P254326		DT868229	up	—	2.27
A_15_P413775			up	—	2.14
A_15_P160571	top1l	NM_001044324	down	—	3.62
A_15_P116538	ppm1g	NM_201488	down	—	3.18
A_15_P503787		XM_690528	down	—	2.26
A_15_P512832			up	—	2.02
A_15_P197796	si:dkey-188i13.1	BC090712	down	—	2.37
A_15_P102642	si:ch211-59d15.8	NM_001164029	down	—	2.26
A_15_P207256	otx1l	NM_131215	up	—	2.08
A_15_P513977			up	—	2.02
A_15_P184566	cad	NM_001009884	up	—	2.43
A_15_P164431	zgc:136892	NM_001040360	up	—	2.19
A_15_P114320			up	—	2.09
A_15_P446420	zgc:136689	NM_001045312	down	—	2.10
A_15_P204133		XM_001344601	up	—	5.96
A_15_P156966	LOC562675	XM_686050	down	—	4.11
A_15_P539272			down	—	2.13
A_15_P178551		BC142739	down	—	2.59
A_15_P103593	mgll	NM_200297	down	—	4.31
A_15_P484495		XM_692771	down	—	5.30
A_15_P284371	upp1	NM_001013301	down	—	2.38

A_15_P339060			up	—	2.01
A_15_P105601	zgc:152816	NM_001077760	down	—	2.58
A_15_P532737			up	—	3.22
A_15_P192806	zgc:100856	NM_001003536	up	—	2.21
A_15_P410650		AY968596	up	—	2.16
A_15_P116045	zgc:136689	NM_001045312	down	—	2.30
A_15_P535082			down	—	4.89
A_15_P382895			down	—	2.25
A_15_P152486	zgc:152896	NM_001077534	down	—	5.36
A_15_P198006	LOC100004967	XM_001340075	down	—	2.16
A_15_P309786			up	—	2.84
A_15_P199016	zgc:100957	NM_001003620	down	—	2.68
A_15_P570517	ihha	NM_001034993	down	—	2.04
A_15_P298691	LOC564478	XM_687812	down	—	3.38
A_15_P281316	zgc:153154	NM_001045413	up	—	2.42
A_15_P104030	lect1	NM_001126448	up	—	2.12
A_15_P186201	LOC562296	XM_685694	up	—	2.54
A_15_P118927	gpr177	NM_213146	up	—	2.01
A_15_P158326	nipbl	NM_001161447	down	—	2.65
A_15_P558247	LOC566967	XM_690250	up	—	2.00
A_15_P474340			up	—	2.40
A_15_P120021	upf3b	NM_200954	down	—	2.74
A_15_P116455	jag1b	NM_131863	down	—	2.31
A_15_P189646	LOC553504	BC091795	down	—	2.62
A_15_P184581	zgc:85746	NM_214819	down	—	2.47
A_15_P134976		BC098606	down	—	3.73
A_15_P198711	si:dkey-223d7.5	NM_001163298	down	—	3.34
A_15_P407915	si:ch211-145m1.1	NM_001130781	down	—	2.08
A_15_P562577			down	—	2.04
A_15_P167196			up	—	2.08
A_15_P175571	syntaxin1b	NM_131523	down	—	3.88
A_15_P116024		BC091977	down	—	3.03
A_15_P412395	zgc:163030	NM_001089413	up	—	6.14
A_15_P202106	LOC570106	XM_693543	down	—	5.16
A_15_P190141	zgc:85787	NM_213167	down	—	3.50
A_15_P188616		XM_001338077	down	—	10.43
A_15_P185121	im:7140055	XM_679746	up	—	2.26
A_15_P461090			down	—	2.36
A_15_P190771	ahnak	BC124733	down	—	4.24
A_15_P435990			down	—	5.16
A_15_P156036	zgc:152774	NM_001075109	down	—	2.78
A_15_P154156	zgc:113362	NM_001014310	down	—	3.51
A_15_P108891	zgc:100896	NM_001003524	down	—	2.53

A_15_P161851	zgc:158707	NM_001080638	down	—	4.74
A_15_P186781	LOC100150828	XM_001919699	down	—	7.53
A_15_P190786	hmmr	NM_199580	down	—	2.64
A_15_P499637	csf2rb	NM_001113733	down	—	2.55
A_15_P101060	hic1l	NM_131483	down	—	2.80
A_15_P237121	ppp1r12a	NM_001003870	down	—	3.88
DCP_1_1			—	—	2.11
A_15_P216631			down	—	2.11
A_15_P109100	ncf1	NM_001030071	down	—	2.58
A_15_P320701	fam160b2	NM_001077766	down	—	3.25
A_15_P117714		BC059206	down	—	2.59
A_15_P180981	cadm1a	NM_001113551	down	—	6.55
A_15_P117002	zgc:112994	NM_001020707	down	—	2.71
A_15_P191206			down	—	4.60
A_15_P121073	im:6896251	XM_685145	down	—	2.80
A_15_P212191	cpsf1	NM_001114681	down	—	3.06
A_15_P198456	DKEY-87L9.2	XM_677653	down	—	5.13
A_15_P194161	zgc:112431	NM_001017782	down	—	2.22
A_15_P178246		BC134197	down	—	2.76
A_15_P145086	neo1	NM_173218	down	—	2.73
A_15_P434245	antxr2a	NM_001044709	down	—	2.22
A_15_P180526	LOC553309	BC090800	down	—	2.58
A_15_P114476	im:7141573	CK680095	down	—	2.42
A_15_P202551	baz1a	BC096971	down	—	4.43
A_15_P104868	LOC100003999	XM_001343386	up	—	2.11
A_15_P133296	rhoaa	NM_213137	down	—	2.03
A_15_P106003			down	—	2.79
A_15_P241696		CT711882	up	—	2.29
A_15_P298146	LOC797886	XM_001338312	down	—	11.51
A_15_P102338	trmt5	NM_001145583	up	—	2.17
A_15_P110542	tbx3b	NM_001101670	down	—	2.19
A_15_P172646	cldng	NM_180965	up	—	2.17
A_15_P118326	efna1a	NM_200597	down	—	2.80
A_15_P130496		AB119258	up	—	2.35
A_15_P178761	wu:fi14a03	BC146727	down	—	2.09
A_15_P592027	wu:fb30e12	CN323290	down	—	2.19
A_15_P186851		DQ017638	down	—	2.47
A_15_P384325	haus6	NM_001079974	up	—	2.05
A_15_P157431	LOC100002543	XM_001342265	down	—	3.65
A_15_P141871		BC091840	down	—	3.63
A_15_P134441	pdcd11	NM_001089368	down	—	2.83
A_15_P178041		BC129399	down	—	2.68
A_15_P102964	ppl	NM_001137666	down	—	2.90

A_15_P592157	mgll	NM_200297	down	—	3.06
A_15_P118745			up	—	6.21
A_15_P196916		XR_044976	down	—	4.33
A_15_P443590			down	—	5.64
A_15_P396625			up	—	2.72
A_15_P186026	shq1	NM_001080600	down	—	3.02
A_15_P121196	LOC561766		down	—	4.40
A_15_P563677	zgc:55443	NM_213502	down	—	2.02
A_15_P173771	prkcq	NM_001089370	down	—	2.72
A_15_P337395			up	—	5.83
A_15_P261276			down	—	4.31
A_15_P200651	LOC565975	EH436377	up	—	2.05
A_15_P178222		BC134122	down	—	2.48
A_15_P173676	daam1l	NM_001030136	down	—	2.01
A_15_P163151	zgc:162265	NM_001089326	up	—	2.06
A_15_P572142	zgc:100960		up	—	2.64
A_15_P200091	rbpja	BC092918	down	—	2.13
A_15_P136341		BC090794	down	—	2.28
A_15_P161821	zgc:162874	NM_001099252	down	—	3.10
A_15_P113913		XM_001339115	down	—	8.62
A_15_P179951	col6a2	BC134051	down	—	2.11
A_15_P206491	ihha	NM_001034993	down	—	2.17
A_15_P156676	zgc:175268	NM_001114462	down	—	3.15
A_15_P206001	zgc:101621	NM_001006093	up	—	2.69
A_15_P134746		BC095094	down	—	2.10
A_15_P181371	LOC100005116	XM_001338974	down	—	2.14
A_15_P181261	lars	XM_693187	up	—	2.20
A_15_P105183		BC064299	up	—	2.01
A_15_P262346			down	—	2.68
A_15_P177106	si:dkey-67c22.2	NM_001130400	down	—	2.43
A_15_P439010	gldc	NM_199554	down	—	4.03
A_15_P141921	zgc:77593	NM_200880	down	—	2.11
A_15_P114066	hoxa13b	NM_131194	up	—	2.08
A_15_P177066		BC107512	down	—	3.33
A_15_P267626			down	—	2.35
A_15_P196051	grk1a	NM_001034181	down	—	2.44
A_15_P100675			down	—	2.43
A_15_P185441	xirp2	BC133989	down	—	2.56
A_15_P178331	tpr	NM_001030123	down	—	4.47
A_15_P118608	mpdu1a	NM_001004545	up	—	2.11
A_15_P200796		XM_695597	down	—	2.72
A_15_P156022	nrxn3b	NM_001080047	down	—	2.35
A_15_P274441	anp32a	NM_199986	down	—	2.44

A_15_P115483	LOC558832	BC154646	up	—	2.73
A_15_P108194	pak2a	NM_001002717	down	—	2.28
A_15_P206521	si:dkey-218n20.1	NM_001045026	down	—	2.45
A_15_P348510			up	—	2.01
A_15_P119540	wu:fj84d10	CK015579	down	—	3.13
A_15_P107240	LOC100001225	XM_001337395	up	—	2.91
A_15_P137356			up	—	2.11
A_15_P121294	zgc:123105	NM_001039994	down	—	2.01
A_15_P183181	zgc:171857	NM_001122628	down	—	2.62
A_15_P285701	ptgs2a	NM_153657	up	—	2.32
A_15_P190971		XM_681489	down	—	2.69
A_15_P184286	si:ch211-199m3.9	NM_001110519	down	—	3.33
A_15_P184510			up	—	20.30
A_15_P513912	CH211-279L9.6	XM_678293	down	—	2.17
A_15_P134426		BC091912	down	—	2.07
A_15_P401090	zgc:92599	NM_001004607	up	—	2.32
A_15_P190816	slc7a2	NM_001008584	up	—	2.06
A_15_P541427	dnl2	NM_200099	down	—	3.67
A_15_P106441	si:dkey-151c10.1	NM_001100032	up	—	2.10
A_15_P212091		BC076069	up	—	2.14
A_15_P142841	osr2	NM_001017694	down	—	2.73
A_15_P173176	zgc:152774	NM_001075109	down	—	2.92
A_15_P210961	rrm2	NM_131450	up	—	2.27
A_15_P130356	tpr	NM_001030123	down	—	2.61
A_15_P101546	crim1	NM_212821	down	—	2.23
A_15_P202116	im:6906849	BC076425	down	—	2.97
A_15_P509447			down	—	4.31
A_15_P237456	wu:fi27h03	XM_679489	down	—	3.03
A_15_P384760			down	—	4.23
A_15_P277031			up	—	4.31
A_15_P133501	sema6d	NM_212999	down	—	2.45
A_15_P220766			down	—	3.65
A_15_P180541	LOC100004739	XM_001343932	up	—	2.35
A_15_P165321	LOC565839	XM_689101	down	—	2.12
A_15_P139211		XM_680821	down	—	2.68
A_15_P110224		CT698302	down	—	5.68
A_15_P105630	fancl	NM_212982	up	—	2.45
A_15_P104047			down	—	2.93
A_15_P156131	tbx3b	NM_001101670	down	—	2.54
A_15_P114064	insig2	NM_212804	down	—	2.01
A_15_P201836	si:dkey-67c22.2	NM_001130400	down	—	2.58
A_15_P346585			up	—	2.58
A_15_P133966	tlk1b	NM_001024951	down	—	2.12

A_15_P139556	cdc14b	NM_200179	down	—	2.06
A_15_P191581	LOC571401	XR_029888	down	—	3.69
A_15_P120089	sgcg	NM_001003748	down	—	3.11
A_15_P164976	LOC795748	XM_001335976	down	—	2.34
A_15_P131941		AF210648	down	—	2.19
A_15_P112284	ppp4cb	NM_001111168	up	—	2.56
A_15_P349585	ahnak	XM_682604	down	—	2.31
A_15_P328911		CT652973	down	—	5.70
A_15_P146341	LOC566287	XM_689556	down	—	2.61
A_15_P506652	myom1a	NM_001161340	down	—	3.45
A_15_P178821	mnta	BC150240	down	—	2.03
A_15_P413905	LOC793098	XM_001332041	down	—	2.12
A_15_P169326	ccdc146	BC124200	down	—	2.38
A_15_P106465	adam10a	NM_001159314	down	—	2.05
A_15_P112892		BC091988	down	—	2.46
A_15_P537412	strn3	BC142862	down	—	3.96
A_15_P121285	ptrf	NM_001114549	down	—	4.15
A_15_P163311	ncapg2	NM_001089492	up	—	2.01
A_15_P209986	dhdhl	NM_214735	down	—	2.35
A_15_P162596	zgc:152864	NM_001077764	down	—	2.55
A_15_P219486			down	—	2.04
A_15_P177111	lyricl	NM_001007135	down	—	2.65
A_15_P440970	gclc	NM_199277	down	—	2.54
A_15_P182016	iffo1	XM_685073	down	—	4.62
A_15_P391300			down	—	2.53
A_15_P186404		BC079480	down	—	2.03
A_15_P183476	LOC100002572	BC095146	down	—	4.25
A_15_P105198	ddx42	NM_001037805	down	—	2.65
A_15_P120760	si:dkey-231l1.6	NM_001080992	down	—	2.37
A_15_P112816	LOC100147996	XM_001921128	up	—	2.00
A_15_P456865		CK028289	up	—	2.01
A_15_P112719	zgc:92428	NM_001003732	up	—	2.14
A_15_P106837	zgc:114180	NM_001030248	down	—	2.49
A_15_P107840	marveld2b	NM_001126406	up	—	2.55
A_15_P102278		EH605325	up	—	2.23
A_15_P107652	ptgs2a	NM_153657	up	—	2.20
A_15_P449880			up	—	2.19
A_15_P113818	sb:cb627	BC115332	down	—	2.63
A_15_P181981	setdb1a	NM_001044767	down	—	2.57
A_15_P115680			up	—	2.30
A_15_P198576	zgc:152779	NM_001077535	down	—	2.04
A_15_P500157			up	—	3.30
A_15_P101624	phf8	XM_689807	down	—	2.34

A_15_P263811		AW128375	down	—	2.03
A_15_P430090		EB842738	down	—	2.60
A_15_P106434			down	—	4.99
A_15_P345745			up	—	2.57
A_15_P108524	prph	NM_131054	up	—	2.31
A_15_P194836	shox	NM_001126411	down	—	2.71
A_15_P198901	klhl20	NM_213001	down	—	2.08
A_15_P239116	ap4b1l	NM_200338	down	—	2.15
A_15_P162441	smc1al	NM_212810	down	—	2.74
A_15_P178891	myhb	BC150415	down	—	3.22
A_15_P287161			down	—	3.79
A_15_P178791	hectd1	NM_001002504	down	—	2.86
A_15_P434305			up	—	2.36
A_15_P178801	si:dkey-147f20.5	NM_001161377	up	—	2.23
A_15_P136751	LOC558640	XM_681870	down	—	2.01
A_15_P173826	unc119.2	NM_001040394	down	—	2.03
A_15_P437055		BC107512	down	—	2.70
A_15_P197716		XR_044895	down	—	2.62
A_15_P146976		BC152291	down	—	2.07
A_15_P181786	daxx	NM_001044949	down	—	2.26
A_15_P536237	wu:fc07e08	XM_680061	down	—	5.47
A_15_P162901	zgc:153738	NM_001079968	down	—	2.95
A_15_P114529	si:ch211-129c21.6	NM_001045015	down	—	2.02
A_15_P390725		CT607160	up	—	2.39
A_15_P322966	pcna	NM_131404	up	—	2.08
A_15_P178981	cxxc1l	NM_200599	down	—	3.64
A_15_P244746	LOC560644	XM_684039	up	—	5.90
A_15_P481085		CT700592	up	—	2.67
A_15_P112851	fpgs	NM_213437	down	—	2.46
A_15_P144161	LOC568230	XM_691549	down	—	2.59
A_15_P188826	CH73-250D21.2	NM_001126386	down	—	2.78
A_15_P186036	rbm19	NM_198915	down	—	3.25
A_15_P301341	LOC571620	XM_701503	down	—	2.12
A_15_P144656	LOC100000576	XM_001337087	down	—	2.22
A_15_P211181	zgc:114180	NM_001030248	down	—	2.66
A_15_P104664	arr3l	NM_001002405	down	—	2.12
A_15_P168076			up	—	2.74
A_15_P198981			down	—	5.16
A_15_P402910		DN894231	down	—	2.30
A_15_P111768	adra2b	NM_207638	down	—	2.86
A_15_P105540	zgc:66483	NM_199825	up	—	2.02
A_15_P102124	adhfe1	NM_207086	up	—	2.15
A_15_P513617	ahnak	BC124733	down	—	2.42

A_15_P119425	cbl	NM_001007330	down	—	2.01
A_15_P270516		XM_001344147	down	—	2.39
A_15_P146061	zgc:165373	XM_685461	down	—	3.07
A_15_P263296		EV558642	down	—	3.17
A_15_P261926			down	—	2.32
A_15_P101440			up	—	3.22
A_15_P267381			down	—	2.97
A_15_P598767	irx1b	NM_131823	up	—	2.38
A_15_P446335	LOC558416	XM_681621	down	—	3.13
A_15_P170136	zgc:56231	NM_213188	up	—	2.24
A_15_P560357	ccrl1a	XM_001342755	up	—	2.03
A_15_P495672			up	—	2.30
A_15_P258606	LOC100007727	XM_001346072	down	—	2.67
A_15_P408265	zgc:162197	NM_001110454	down	—	2.02
A_15_P134241		BC090500	down	—	2.15
A_15_P109612	cldna	NM_131762	up	—	2.09
A_15_P106950		BC079483	down	—	2.84
A_15_P319641			down	—	3.05
A_15_P295321			up	—	2.02
A_15_P100058	zgc:152845	NM_001077572	down	—	3.99
A_15_P318656			up	—	3.23
A_15_P185746	zranb2		down	—	3.13
A_15_P114416	fancl	NM_212982	up	—	2.13
A_15_P153811	zgc:153018	NM_001076639	down	—	2.05
A_15_P101297			down	—	2.40
A_15_P445565		XM_001345940	down	—	3.72
A_15_P599387	wu:fb94h07	CK693674	up	—	2.07
A_15_P567452	cdx1a	NM_212836	down	—	2.15
A_15_P117061			down	—	3.76
A_15_P433260	si:dkey-8l13.4	NM_001044986	down	—	2.57
A_15_P181226			down	—	2.19
A_15_P355380			down	—	2.37
A_15_P117909	fzd9	NM_131511	up	—	2.00
A_15_P282511			up	—	2.19
A_15_P264796			down	—	2.28
A_15_P113852	ftr76	BC155657	down	—	7.57
A_15_P493732	rock2b	NM_001093747	down	—	3.14
A_15_P177056	si:ch211-284a13.1	BC107500	down	—	3.58
A_15_P209371	zc3hdc1l	BC085328	down	—	2.05
A_15_P115330	LOC563198	XM_686560	up	—	2.51
A_15_P109078	wu:fb81h03	XM_689244	down	—	3.22
A_15_P106928	calcoco1	NM_001110166	down	—	4.16
A_15_P115076	wu:fi25h02	BC055503	down	—	2.92

A_15_P182386	zgc:153912	NM_001079971	down	—	3.65
A_15_P150961	zgc:162618	NM_001089331	up	—	2.36
A_15_P261246			down	—	3.58
A_15_P142396	LOC562205	NM_001044980	up	—	8.97
A_15_P230191	zgc:112071	NM_001017646	up	—	2.17
A_15_P473275	kri1l	NM_001002041	down	—	2.11
A_15_P106287	snapc1b	NM_200254	up	—	2.30
A_15_P106206	im:7159195	XM_684566	down	—	2.17
A_15_P171411	tmprss4a	NM_001077738	up	—	2.19
A_15_P171571	zgc:113320	NM_001017910	down	—	3.15
A_15_P600537	daam1	XM_702261	down	—	2.28
A_15_P137061	LOC794759	XM_001922883	down	—	2.06
A_15_P419440			down	—	2.30
A_15_P106730	nudt4	NM_200110	up	—	2.55
A_15_P597357	cfl1	NM_213639	up	—	2.40
A_15_P420580	si:ch73-30n17.4	XM_696010	down	—	3.55
A_15_P191566			down	—	2.51
A_15_P197956	sfrp5	NM_131858	up	—	2.46
A_15_P419955	rcc1	NM_213178	up	—	2.16
A_15_P178356	LOC557176	BC135013	down	—	2.41

Table 2

50 top GO processes represented in MetaCore (FDR< 0.05.

Process	**p-value**
cellular process	9.40e-17
developmental process	5.82e-13
system development	3.78e-13
multicellular organismal development	2.42e-13
anatomical structure development	1.94e-13
muscle system process	3.62e-12
muscle contraction	1.13e-11
response to hormone stimulus	7.27e-10
regulation of striated muscle cell differentiation	3.25e-10
anatomical structure formation involved in morphogenesis	9.79e-09
regulation of muscle cell differentiation	9.63e-09
response to endogenous stimulus	7.79e-09
regulation of multicellular organismal process	4.68e-09
tissue development	4.65e-09
muscle structure development	4.35e-09
anatomical structure morphogenesis	3.74e-09

regulation of localization	3.45e-09
organ development	3.13e-09
vasculature development	2.97e-09
cellular component organization	1.82e-09
localization	1.66e-09
blood vessel development	1.61e-09
positive regulation of biological process	1.57e-09
regulation of cell differentiation	9.54e-08
cellular metabolic process	9.26e-08
regulation of transport	8.17e-08
multicellular organismal process	7.52e-08
regulation of muscle contraction	7.52e-08
positive regulation of muscle contraction	7.38e-08
regulation of muscle system process	6.98e-08
epithelium development	6.15e-08
regulation of skeletal muscle tissue development	5.31e-08
response to organic substance	4.68e-08
regulation of vasoconstriction	4.61e-08
cellular developmental process	4.13e-08
macromolecule modification	3.92e-08
muscle organ development	3.54e-08
establishment of localization	3.35e-08
response to steroid hormone stimulus	2.50e-08
regulation of system process	2.01e-08
cytoskeleton-dependent intracellular transport	1.93e-08
transport	1.68e-08
cell differentiation	1.40e-08
nervous system development	2.17e-07
organ morphogenesis	1.92e-07
organelle organization	1.60e-07
embryonic development	1.50e-07
post-translational protein modification	1.42e-07
regulation of developmental process	1.39e-07
protein modification process	1.06e-07

Table 3

15 top maps and pathways represented in MetaCore (FDR< 0.05).

Pathway	GeneGo Map Folders	Cell Process	p-Value
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodelling Cell adhesion_Gap junctions Development_CNTF receptor signaling	**Tissue remodeling and wound repair**	Cell communication; Protein amino acid phosphorylation	1.068e-7
Immune response_IL-1 signaling pathway Immune response_IL-6 signaling pathway Development_Transcription regulation of granulocyte development Immune response Immunological synapse formation	**Immune system response**	Positive regulation of smooth muscle cell proliferation; JAK-STAT cascade involved in growth hormone signaling pathway	2.344e-6
Immune response_IL-1 signaling pathway Cell adhesion_Chemokines and adhesion Immune response_IL-6 signaling pathway	**Inflammatory response**	Positive regulation of smooth muscle cell proliferation; Cell migration	9.541e-5
Development_TGF-beta-dependent induction of EMT via MAPK Development_Regulation of epithelial-to-mesenchymal transition (EMT) Development_CNTF receptor signalling	**Cell differentiation**	Positive regulation of apoptosis; Microtubule cytoskeleton organization	1.099e-5
DNA damage_Nucleotide excision repair DNA damage_Role of Brca1 and Brca2 in DNA repair Apoptosis and survival_Role of IAP-proteins in apoptosis	**DNA-damage response**	Response to DNA damage stimulus	8.560e-4
Cell cycle_ESR1 regulation of G1/S transition DNA damage_ATM/ATR regulation of G1/S checkpoint Cell cycle_Role of SCF complex in cell cycle regulation	**Cell cycle and its regulation**	Cell cycle	2.612e-4
Apoptosis and survival_Ceramides signaling pathway DNA damage_Role of SUMO in p53 regulation Development_EPO-induced Jak-STAT pathway	**Apoptosis**	Apoptosis and survival	7.757e-3
Blood coagulation_GPIb-IX-V-dependent platelet activation Blood coagulation_GPVI-dependent platelet activation Blood coagulation_GPCRs in platelet aggregation	**Blood clotting**	Hemostasis	7.252e-3
Translation_Non-genomic (rapid) action of Androgen Receptor Translation _Regulation of EIF2 activity Translation_Translation regulation by Alpha-1 adrenergic receptors Translation_Insulin regulation of translation	**Protein Synthesis**	Translation	4.824e-3

Transcription_CREB pathway Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis Apoptosis and survival_NO synthesis and signaling	**Calcium signaling**	Protein amino acid phosphorylation; Insulin-like growth factor receptor signaling pathway	4.721e-3
Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity Cytoskeleton remodeling_Alpha-1A adrenergic receptor-dependent inhibition of PI3K Muscle contraction_ GPCRs in the regulation of smooth muscle tone	**Vasoconstriction**	Phosphoinositide phosphorylation; Cell adhesion	1.661e-3
Transcription_Receptor-mediated HIF regulation Development_FGFR signaling pathway Development_FGF-family signaling	**Vascular development (Angiogenesis)**	Cell proliferation	1.355e-3
Signal transduction_PTEN pathway Development_IGF-1 receptor signaling Development_CNTF receptor signaling	**Mitogenic signaling**	Cell proliferation	1.184e-3
Proteolysis_Putative SUMO-1 pathway Development_WNT signaling pathway. Part 1. Proteolysis_Putative ubiquitin pathway	**Protein degradation**	Proteolysis	4.080e-2
Translation_Non-genomic (rapid) action of Androgen Receptor Transcription_Androgen Receptor nuclear signaling	**Androgen signaling**	Insulin-like growth factor receptor signaling pathway; Response to estrogen stimulus	3.289e-2